University of New Hampshire
University of New Hampshire Scholars' Repository

Doctoral Dissertations | Student Scholarship

Summer 1973

THE OCCURRENCE OF CHOLINE IN THE MARINE DINOFLAGELLATE, AMPHIDINIUM CARTERI

RICHARD FRANCIS TAYLOR
University of New Hampshire, Durham

Follow this and additional works at: https://scholars.unh.edu/dissertation

Recommended Citation
TAYLOR, RICHARD FRANCIS, "THE OCCURRENCE OF CHOLINE IN THE MARINE DINOFLAGELLATE, AMPHIDINIUM CARTERI" (1973). *Doctoral Dissertations*. 2353.
https://scholars.unh.edu/dissertation/2353

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.

INFORMATION TO USERS

This dissertation was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

A Xerox Education Company

73-4366

TAYLOR, Richard Francis, 1946-
THE OCCURRENCE OF CHOLINE IN THE MARINE DINOFLAGELLATE AMPHIDINIUM CARTERI.

University of New Hampshire, Ph.D., 1973
Chemistry, biological

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED.

THE OCCURRENCE OF CHOLINE IN THE MARINE DINOFLAGELLATE *AMPHIDINIUM CARTERI*

BY

RICHARD F. TAYLOR

B.S., St. Lawrence University, 1968.

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Biochemistry

June, 1972

This thesis has been examined and approved.

Miyoshi Ikawa

Thesis director, Miyoshi Ikawa, Prof. of Biochemistry

William R. Chesbro

William R. Chesbro, Prof. of Microbiology

Galen E. Jones

Galen E. Jones, Prof. of Microbiology

Gerald L. Klippenstein

Gerald L. Klippenstein, Assoc. Prof. of Biochemistry

Samuel C. Smith

Samuel C. Smith, Prof. of Biochemistry and Animal Sciences

August 1, 1972

Date

PLEASE NOTE:

Some pages may have

indistinct print.

Filmed as received.

University Microfilms, A Xerox Education Company

ACKNOWLEDGEMENTS

It has often been said that one cannot truly appreciate an experience until that experience is lost and can only be viewed and reflected upon from a distance. Thus, although I now express my sincere gratitude to Dr. Miyoshi Ikawa for his patience, guidence and friendship in this project and throughout my graduate program, I am sure that I will not fully appreciate his influence on my career until I can reflect back on these years from another point in time.

I was supported throughout this research by my hard-working wife, Nancy, as well as by an NDEA Fellowship.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF SCHEMES

LIST OF FIGURES

ABSTRACT

THE OCCURRENCE OF CHOLINE IN THE MARINE DINOFLAGELLATE *AMPHIDINIUM CARTERI*

by

RICHARD F. TAYLOR

A study was made of suspected choline compounds in the marine dinoflagellate *Amphidinium carteri*. Initial assays of the cultured and lyophilized alga utilizing a choline-requiring strain of *Neurospora crassa* showed that the alga contained approximately 0.38% dry weight choline chloride equivalent. Upon division of this choline chloride equivalence into lipoidal and non-lipoidal forms of choline present in the dinoflagellate by utilization of an ethanolic extraction method followed by butanol partition, almost all of the choline activity in the alga was found due to non-lipoidal forms of choline. Since non-lipoidal forms of choline may act as potential neurotoxins in higher animals, the choline compounds occurring in *A. carteri* were subjected to further analysis.

The choline components of *A. carteri* were ethanol extracted and purified by preparative paper chromatography and precipitation methods. Three non-lipoidal choline components were found to occur in the alga and were designated Unknown Components #1, #2 and #3. Utilizing a number of chemical, physical and chromatographic methods, Unknown Component #1 was found to be a choline ester. Acid hydrolysis of the unknown led to two products, choline chloride, as determined by paper chromatography, and acrylic acid, as determined by gas-liquid chromatography. Thus, the unknown was tentatively identified as acrylylcholine. Positive proof of this

tentative identification of Unknown Component #1 was achieved upon the co-chromatography of the unknown with authentic acrylylcholine in a number of paper and thin-layer systems.

Unknown Component #2 was also shown to be a choline ester by chemical, physical and chromatographic methods. Acid hydrolysis of the unknown again led to two products, choline chloride and an unknown volatile hydrolysis product which was not able to be identified by gas-liquid chromatographic or spectral analysis. Thus, Unknown Component #2 is theorized as being a choline ester with a two or three carbon acyl group. A discussion is included which postulates further that the acyl group of this unknown ester may contain heteroatom substitution and/or may be derived from acrylylcholine.

Unknown Component #3 was found to co-chromatograph in all paper and thin-layer systems with authentic choline O-sulfate. In addition, the spectral, chemical and physical methods applied to the unknown and authentic choline O-sulfate proved conclusively that Unknown Component #3 is choline O-sulfate.

The crude ethanolic extract from A. carteri cells as well as purified Unknown Component #1 proved pharmacologically active in a number of animal systems specifically sensitive to choline esters including clam, crustacean and frog heart assays and mouse intestinal strip assay. In addition, the extracts and whole cells of A. carteri proved potentially toxic to fish and mice in preliminary studies. Based on these results, a discussion is included concerning the distribution, chemical nature, biological activity and possible functions of choline substances and other quaternary nitrogen compounds in marine organisms.

It is concluded that the occurrence of potentially toxic choline substances in a marine dinoflagellate may influence the distribution and passage of these compounds through the marine food chain. Thus, the further analysis of other toxic dinoflagellates for quaternary nitrogen bases utilizing the methods developed in this study is deemed warranted.

I. INTRODUCTION

A. Historical Background.

In 1849, Strecker (118) isolated an organic base from hog bile which he re-examined in 1862 and characterized as an "ethylene-oxide trimethylamine" and which he named "choline" (119). In the meantime, Babo and Hirschbrunn had isolated a new organic base by alkaline hydrolysis of an alkaloid derived from white mustard (Sinapis alba) seed and which they named "sinkalin" (13). The similarities of sinkalin and choline were noted by later investigators and finally the two were proven to be the same compound, choline hydroxide (trimethyl-β-hydroxyethyl-ammonium hydroxide), by Baeyer (16,17) and Wurtz (143,144) who chemically characterized and synthesized the substance. The studies by Baeyer also clarified the confusion resulting from Liebreich's work with an organic base isolated from hydrolyzed brain "protagon" (lecithin) which he had named neurine (82). This "neurine" was shown by Baeyer to be choline and the name "neurine" was reserved for vinyltrimethylammonium hydroxide, a decomposition product of choline.

During his work on the chemical characterization of choline, Baeyer synthesized a new derivative of choline by treatment of choline chloride with acetyl chloride:

$$Cl^-(CH_3)_3\overset{+}{N}CH_2CH_2OH + CH_3COCl \longrightarrow Cl^-(CH_3)_3\overset{+}{N}CH_2CH_2OCOCH_3 + HCl$$

Although the major aim of this experiment was to prove that choline contained an alcoholic hydroxyl group, the resulting product of the reaction, acetylcholine (ACh), has turned out to be a compound of great importance in the fields of biochemistry and physiology.

The study of choline and its esters did not attract much additional interest after these early experiments until Ewins discovered the natural occurrence of acetylcholine in ergot (the ascomycete, Claviceps purpurea) (44), and Dale defined the muscarinic and nicotinic effects of acetylcholine and other choline esters on nerve preparations based on his own observations and earlier work carried out by Hunt and Taveau (34,70). In 1921, a milestone experiment in acetylcholine research was reported by Loewi who showed that parasympathetic nerves produce their stimulation effects by peripheral release of "Vagusstoff," a choline ester "resembling" acetylcholine (85). In this report, Loewi showed that the frog vagus nerve inhibits the frog's heart by liberating this choline ester. After this initial indication of the transmitter function of acetylcholine, a series of studies followed which proved that acetylcholine is indeed a neurohumor (see, e.g., 35,36,47) and is found in a great variety of animal tissues (21, 22,31).

Since these early studies with choline compounds, there has been a continuing interest in them due to their highly active nature in animal neural systems, their possible role as transport factors in lower organisms and plants, and the newer implications of their role as neurotoxins. The present work is concerned with the natural occurrence and function of choline compounds, especially in marine organisms.

B. Naturally-Occurring Choline Compounds.

Since the original discoveries of choline in hog bile (118) and acetylcholine in ergot (44), numerous choline compounds have been found in natural sources as is shown in TABLE 1. Choline itself is a fairly ubiquitous compound in both the plant and animal kingdoms, occurring in all phyla al-

TABLE 1.

Non-Phospholipid, Naturally-Occurring, Choline-Containing Substances.

Name	Structure	Occurrence	References
Choline (chloride)	$HOCH_2CH_2\overset{+}{N}(CH_3)_3$		
Acetylcholine	$CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3$	Echinoderms; Mollusks; Arthropods; Chordates	30, 61, 77, 87, 98
Propionylcholine	$CH_3CH_2COOCH_2CH_2\overset{+}{N}(CH_3)_3$	Mollusks; Mammals	14, 137
Butyrylcholine	$CH_3CH_2CH_2COOCH_2CH_2\overset{+}{N}(CH_3)_3$	Mammals	69
γ -Aminobutyrylcholine	$H_2NCH_2CH_2CH_2COOCH_2CH_2\overset{+}{N}(CH_3)_3$	Mammals	76, 81

TABLE 1 (Continued).

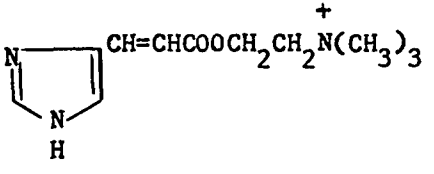
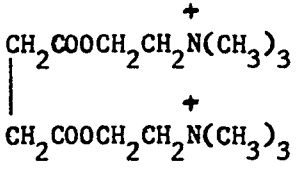
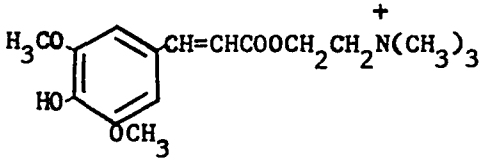
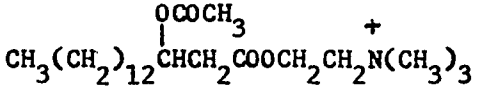
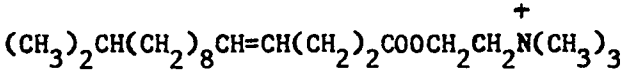
Name	Structure	Occurrence	References
Acrylylcholine	$H_2C{=}CHCOOCH_2CH_2\overset{+}{N}(CH_3)_3$	Mollusks	137
β,β-Dimethylacrylylcholine	$(CH_3)_2C{=}CHCOOCH_2CH_2\overset{+}{N}(CH_3)_3$	Mollusks	77, 136, 137
Urocanylcholine (Murexine)		Mollusks	42, 43, 77, 137, 138
Choline O-sulfate (Sulfurylcholine)	$^{-}O\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}OCH_2CH_2\overset{+}{N}(CH_3)_3$	Bacteria; Fungi; Lichens; Higher Plants; Coelenterates	19, 72, 74, 83, 95, 96, 106
Choline O-phosphate (Phosphorylcholine)	$^{-}O\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{P}}OCH_2CH_2\overset{+}{N}(CH_3)_3$	Bacteria; Higher Plants	74, 86, 126

TABLE 1 (Continued).

Name	Structure	Occurrence	References
Succinyldicholine (Suxamethonium)		?	137, 140
Sinapine		Mustard Seeds	102
Pahutoxin (Ostracitoxin)		Hawaiian Boxfish	25, 124
"Glycolipid" Component		Japanese Oyster sp.	94

though its occurrence in bacteria appears to be the exception rather than the rule (71). Of interest here is the fact that choline in one form or another is found widely among the algae with the exception of the blue-green algae (see 72,73). In most cases in the algae, this choline is probably bound choline in the form of, e.g., phospholipid. Acetylcholine is also fairly ubiquitous but is found chiefly in the animal kingdom (although it has been reported in bacteria (116)). In marine organisms, acetylcholine has been found in echinoderms, e.g., starfish (98); cephalopods, e.g., octopus (61); crustaceans, e.g., lobster (77); and in certain fish, such as in the electric organ of Torpedo (30,87).

Whereas acetylcholine is fairly common in the animal world, other choline esters are not. Propionylcholine (14,137), butyrylcholine (69) and γ-aminobutyrylcholine (76,81) have thus far been reported from only a few natural sources which include chiefly mammalian nervous systems. These compounds are of interest, however, since their corresponding organic acids are also found in mammalian brains and this may suggest a possible neurohumor activity for these compounds with esterase control (see below).

Choline esters with acyl groups derived from acrylic acid are of great interest since they have, thus far, been found only in marine gastropods (although β,β-dimethylacrylylcholine may also be present in the garden tiger moth, Arctia caja (23)). Acrylylcholine has been found in species of Buccinidae (137) while β,β-dimethylacrylylcholine has been reported in the oyster drill, Thias floridana (77,136,137). Urocanylcholine is much more prevalent than the latter two compounds and has been reported in members of Murex, Tritonalia, Urosalpinx, Thias and other gastropod species (42,43,77,137,138). This ester, originally known as murexine, was first located in the hypobranchial (or purple) gland of Murex truculus and char-

acterized as a neuroactive agent by Dubois in 1903 (38). Roaf and Nirenstein (103) in 1907 found and pharmacologically characterized a substance similar to (and probably the same as) murexine from Purpura lapillus.

Other marine organisms have been found to contain unusual choline substances. Pahutoxin, a choline ester unusual in its fat-soluble properties due to its long hydrocarbon acyl group, has been found in the Hawaiian boxfish, Ostracion lentiginosis (25,124) while a component of the "glycolipid" from species of Japanese oysters has been shown to be another long acyl chain choline ester (94).

In addition to these identified choline substances, there have been a number of unidentified substances from marine organisms which have been reported as possessing acetylcholine-like activity in physiological test systems. For example, a substance from the sea hare, Aplysia californica Cooper, has been reported which resembles acetylcholine in its pharmacological properties (141), and homogenates of sea urchin pedicellariae exhibit strong cholinergic activity (93).

Two inorganic esters of choline, choline O-sulfate and choline O-phosphate, are also found to occur naturally. However, their functions appear to differ from the apparent neural functions of organic choline esters. Choline O-sulfate occurs widely in both plants and microorganisms and has been found in bacteria, fungi, algae, lichens and higher plants. In marine organisms, choline O-sulfate has been found in coelenterates (106) while Lindberg has reported the presence of choline O-sulfate as well as taurine, N-methyltaurine and N,N-dimethyltaurine in the red alga Gelidium cartilagineum and has postulated a biosynthetic pathway from the methylated taurines through N,N,N-trimethyltaurine ("taurobetaine," see below) to choline O-sulfate (83). The function of choline O-sulfate in vivo is still uncer-

tain, but the compound appears involved in transport activities. Active uptake mechanisms have been found for the compound in fungi (19) and higher plants (95,96). The compound can also serve as the sole source of sulfur in a number of microorganisms and is utilized by these organisms *via* an active transport system which accumulates the ester within the cell and a sulfatase which hydrolyzes the ester to liberate sulfate for further reduction (19).

Choline O-phosphate has been shown to occur in non-lipoidal forms in both bacteria and plants (74,86,126). The compound has been found to be the major phosphate ester in some plant saps and may act as a phosphate carrier in these saps due to its solubility in organic solvents and its ability to penetrate plant cell membranes (86). The choline compound found in the cell wall of a choline-requiring strain of *Pneumococcus* (126) has been shown to occur in the pneumococcal C-substance of the cell wall and has been characterized as a choline O-phosphate containing ribitol teichoic acid (29).

Finally, two choline compounds which are less well-known and characterized can be mentioned here. Sinapine, the choline ester of sinapic acid, can be extracted from black mustard (*Brassica nigra* Koch) seeds with ethanol (102). It is uncertain whether this compound is the "alkaloid," sinapin, isolated by Babo and Hirschbrunn (13) from white mustard seed (see above), but sinapin would appear to be the same compound as sinapine since the boiling of the former compound with alkali released choline (sinkalin). The other compound to be briefly considered here is succinyldicholine which has not, as yet, been found in a natural source but is of great interest due to its clinical use as a muscle relaxant. It may be postulated that the reason succinyldicholine has not been found *in vivo* is due to its ex-

tremely high lability to hydrolysis (see 140).

C. Pharmacological Actions of Choline and its Esters.

Ever since Loewi (85) theorized in 1921 that acetylcholine might have a transmitter function at neural synapses, the study of choline and its esters has been directed primarily at the functions of these compounds in nervous tissue. Thus, in order to stress the importance of choline compounds isolated from natural sources, a brief discussion of the pharmacological activities of these compounds is in order.

Acetylcholine has been shown to be a neurohumor which stimulates receptors on postsynaptic cell membranes in all neuromuscular, parasympathetic and some sympathetic neural junctions by causing a local depolarization of the postsynaptic cell membrane. The depolarization is then propagated along the postsynaptic receiver cell and results in the response characteristic of that receiver, e.g., striated or smooth muscle contraction. In the normal course of events, the acetylcholine, after having stimulated the receiver cell, is hydrolyzed by acetylcholinesterase to acetic acid and choline (see 135) and thus the receiver cell is able to return back to its polarized or "relaxed" state, ready for further stimulation. Acetylcholine itself is synthesized in presynaptic neural vesicles by choline acetylase (see 62). This is the normal course of acetylcholine excitation. However, if acetylcholine or, as has been recently found, certain other choline esters are present in an animal in amounts such that they cannot be completely hydrolyzed by acetylcholinesterase after stimulating receiver cells, then the receiver cells will remain depolarized, i.e., they cannot return to their polarized or "relaxed" state but, rather, remain in a static "excited" state which results in muscle paralysis, e.g.,

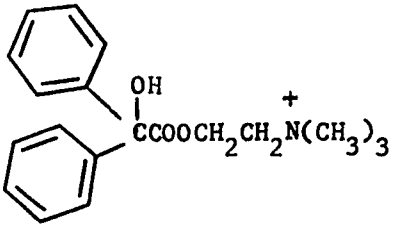
a neuromuscular block. Thus, in effect, choline and certain of its esters have been found to play a dual role in nervous tissue, depending on concentration, either as neurohumors (in low concentrations) or as neurotoxins (in high concentrations). Up to the present time, it has been reported that the naturally-occurring choline esters propionyl-, butyryl-, γ-aminobutyryl-, acrylyl-, β,β-dimethylacrylyl- and urocanylcholine may all exhibit this dual role of neurohumor/neurotoxin similar to acetylcholine depending on concentration as may choline itself (at high concentrations) and succinyldicholine.

The finding that choline esters can act as neurotoxins has led to the postulate that such compounds may act as defensive/offensive substances in organisms in which they are concentrated in high amounts in secretions and non-neural organs. For example, urocanylcholine, which has a neuromuscular blocking action of the typical (high concentration) acetylcholine depolarizing type (137), is found uniquely in the hypobranchial glands of gastropods such as species of Murex. Since the hypobranchial gland of these snails is known to secrete its contents into the surrounding area when the animal is engaged in food-gathering and since the content of urocanylcholine may comprise up to 1.4 mg per gram of organ (58), the choline ester may act as a neurotoxic secretion. Additional evidence for such a theory is the fact that gastropods do not appear to contain acetylcholine as a major neurohumor but rather contain compounds such as γ-aminobutyric acid and glutamic acid as neurohumors (50) and thus might be considered relatively "immune" to the neurotoxic effects of a secreted choline ester.

Evidence also exists for other types of choline toxicity besides such direct neurotoxin effects. Large amounts of free choline (0.1-0.5% dry weight) have been found in the ovaries of the shellfish Callistra brevi-

siphonata and are believed to be responsible for poisonings which have resulted from the ingestion of the shellfish (10). The choline in the ovaries is postulated as arising from the action of esterases on choline esters and presents an interesting question as to the nature and source of these esters.

One last topic must be considered concerning the pharmacological activity of choline esters and this concerns their structure/activity relationships. It has been shown by a number of workers that the ability of choline esters to act as depolarizing transmitters at neural junctions depends on the nature and size of both the acyl portion of the ester and the substituents on the quaternary nitrogen of the choline moiety. If the ester group is eliminated and the chain length of choline reduced so that the compound tetramethylammonium (tetramine) results, only a weak cholinergic (depolarizing) activity remains (9). If the quaternary nitrogen methyl groups of choline are replaced singly or wholly by, e.g., ethyl groups, the resulting choline analog is non-cholinergic and instead becomes anti-cholinergic due to its assuming a role as a competitive antagonist of acetylcholine at neural junctions. Such a competitive antagonist will retain the ability to combine with the receiver cell receptors and prevent the acetylcholine from reaching these receptors but will not have the properties to depolarize the receptor sites and thus stimulate the receiver cell. Curare acts in this manner and thus such neural blockage can be considered "curare-like." A similar loss of cholinergic activity with concomitant gain of anti-cholinergic activity is found when the acyl group of a choline ester is increased in size past butyrylcholine (for straight-chain esters) or past a single aromatic nucleus. Thus, benzilcholine is an extremely potent anti-cholinergic agent and, as expected, shows no depolarizing activity at the receiver cell receptor sites:



Benzilcholine

An exception to this minimum size/activity relationship appears to be β ,β-dimethylacrylylcholine. The branched and unsaturated nature of this compound, however, may effect its steric conformation in such a manner as to explain its cholinergic activity. Similar explanations of steric considerations have been applied to such compounds as urocanyl-, acetyl-β -methyl- and succinylcholine to explain their depolarizing cholinergic activity (9,24,28,31,99,133,134).

D. Other Quaternary Nitrogen Compounds Occurring in Marine Organisms.

Although the primary concern of this report is the characterization of choline compounds from marine sources, there exist other quaternary ammonium compounds which must also be considered due to their widespread occurrence in the marine biosphere. These compounds behave similarly to choline compounds with respect to their chemical and pharmacological properties and lend support to the theory that the quaternary nitrogen functional group in low molecular weight organic bases is responsible for the activity of these compounds in animal neural systems.

TABLE 2 lists the non-choline, quaternary amine compounds which have been reported from marine sources. The first compound, tetramine, is a classic example of a non-choline quaternary ammonium compound which exhibits cholinergic activity in nervous tissue. The activity of tetramine and of many of the other compounds listed in TABLE 2 is usually referred to as

TABLE 2.

Non-Choline, Naturally-Occurring Quaternary Bases in Marine Organisms.

Name	Structure	Occurrence (Phyla)	References
Tetramine ("Thalassin")	$^{+}N(CH_3)_4$	Coelenterata; Mollusca	6, 11, 45, 46, 88, 132
Betaine (Glycine betaine)	$^{-}OOCCH_2\overset{+}{N}(CH_3)_3$	Echinodermata; Mollusca; Annelida; Arthropoda; Chordata	18, 132
γ -Butyrylbetaine	$^{-}OOCCH_2CH_2CH_2\overset{+}{N}(CH_3)_3$	Coelenterata; Mollusca; Echinodermata	1, 78, 132
Taurobetaine	$^{-}O_3SCH_2CH_2\overset{+}{N}(CH_3)_3$	Porifera; Coelenterata	4, 32

TABLE 2 (Continued).

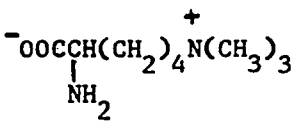
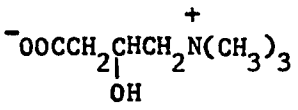
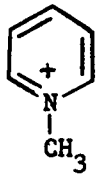
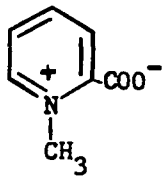
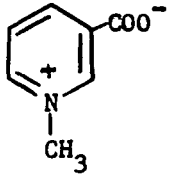
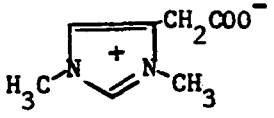
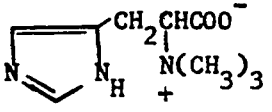
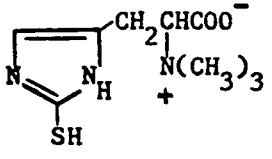
Name	Structure	Occurrence (Phyla)	References
Laminine		Phaeophyta	121
Carnitine		Porifera; Coelenterata; Echinodermata; Mollusca; Annelida; Arthropoda; Chordata	18, 52
N-Methylpyridinium		Echinodermata; Mollusca	7, 33, 78
Homarine		Porifera; Coelenterata; Echinodermata; Mollusca; Arthropoda	2, 3, 54, 65, 78, 88, 89, 132

TABLE 2 (Continued).

Name	Structure	Occurrence (Phyla)	References
Trigonelline		Porifera; Coelenterata; Eichinodermata; Mollusca; Arthropoda	2, 3, 58 62, 132
Zoo-Anemonine		Coelenterata	2, 5, 132
Herzynine		Arthropoda	3
Ergothioneine		Arthropoda	3

"curare-like, depolarizes," i.e., these compounds prevent the normal response of a receptor tissue to acetylcholine (thus, curare-like) and irreversibly depolarize the receptor cells since the receptor sites are "saturated" with the cholinergic compounds. Thus, a compound such as tetramine acts in the same neurotoxic manner as an excess of acetylcholine, or some other choline ester, by preventing the receptor cell from returning to its polarized or "relaxed" state, ready for further stimulation.

The principal studies of tetramine have been accomplished with the marine gastropods, Neptunea anthritica and Neptunea antiqua, snails favored for their flavor among epicureans but which have been implicated in outbreaks of food poisoning (58). The toxin from these organisms is located in their salivary glands, which contain tetramine, histamine, choline and a suspected choline ester (11). The tetramine is believed to be the active factor in the toxin since it may comprise from 7-9 mg per gram of the gland from the Japanese species, N. anthritica (11), and 20-30 mg per gram of the gland from the Swedish species, N. antiqua (45) while the other components are present only in trace amounts in the glands. The toxin from these snails produces typical acetylcholine-like neurointoxication with motor paralysis, respiratory failure, lowering of blood pressure, and depression of heart beat, with possible death from high doses. Tetramine has also been found in coelenterates and may be involved with a protein to produce the toxicosis resulting from nematocyst stings from these organisms (132).

The betaines, a general class of quaternary ammonium bases, are also represented in marine organisms and appear to possess a curare-like, depolarizing action similar to tetramine. The parent of the series, betaine (glycine betaine), has been found in echinoderms, mollusks, annelids, arthropods and chordates, e.g., it has been found in the hypobranchial gland of

the gastropod Thias haemastoma, in the lobster, and in the urochordate Ascidia nigra (18). γ -Butyrylbetaine, the betaine of γ -aminobutyric acid, a powerful convulsant found in mammalian brain tissue (68), is not quite as widespread as betaine but has been reported in coelenterates, mollusks and echinoderms, e.g., in the tentacles of the sea anemone Condylactis gigantea (132) and in the venom duct of species of Conus (78). Taurobetaine, the betaine of taurine, has thus far been reported only in the sponge Geodia gigas (4) and the coelenterate Briareum asbestinum (32) where it is present in concentrations greater than 0.3% dry weight of the organism.

Some straight chain compounds related to the betaines from marine sources include carnitine and laminine. The former substance is almost ubiquitous in animals as a metabolite (51) and in brain tissue (67) and has been reported in almost all phyla of marine organisms, including the porifera, coelenterata, echinodermata, mollusca, annelida, arthropoda and chordata (18,52). Laminine, the betaine of lysine, has been isolated from water extracts of the brown algae, Laminariales, including Laminaria augustata and species of Alaria, Costaria and Eisenia (121). Laminine monocitrate appears to exhibit acetylcholine-like effects on heart and smooth muscle preparations.

N-Methylpyridinium, homarine and trigonelline are all derivatives of pyridine. The latter two compounds are the N-methyl derivatives of picolinic and nicotinic acids, respectively. Although these compounds are not, strictly speaking, quaternary ammonium compounds, their structure and charged nitrogen appear to confer properties and activities on these compounds similar to true quaternary nitrogen compounds. N-Methylpyridinium has been reported in echinoderms and mollusks (7,78,97) while homarine and trigonelline have been found throughout the marine phyla, having been re-

ported in members of the porifera, coelenterata, echinodermata, mollusca and arthropoda (2,3,54,89,132). These compounds act as anti-cholinergics, i.e., they have curare-like activity, but they do not appear to depolarize receiver cell receptor sites as do the betaines and tetramine. Furthermore, they are found throughout the other organs studied in the organisms and not only in the glandular and stinging organs thus suggesting a different function from other quaternary ammonium compounds (see below).

The final series of quaternary ammonium compounds found in marine organisms include the unusual compounds zoo-anemonine, herzynine and ergothioneine. The first compound is the N,N'-dimethylbetaine of imadazole acetic acid and has been extracted from the tentacles of coelenterates (2, 5,132). Herzynine, the betaine of histidine, and ergothioneine, the betaine of 2-thiol-histidine, have been found in only one marine source, the horseshoe crab Limulus polyphemus (3) although the occurrence of the latter compound in other sources is well known (92). The activities of these compounds are still unknown. However, it may be theorized that they may stimulate neural tissues and cause allergenic reactions due to their structural similarities to histamine.

The function of non-choline quaternary ammonium compounds in marine organisms is still not certain. In most cases, similar to choline esters, these compounds are located in glands or tissues which do not function as centers of neural processes but rather as secretory or defensive/offensive organs. Thus, these compounds might be considered as neurotoxins, a theory supported by reports that compounds such as tetramine, homarine, trigonelline, γ-butyrylbetaine and taurobetaine are found in the stinging tentacles of coelenterates (89,127,132) where they may aid in defense and/or feeding, while tetramine, homarine and γ-butyrylbetaine appear to act in

conjunction with a protein to cause the toxic properties of some snail salivary secretions (11,78). However, an alternate theory has been suggested for the function of these compounds as osmotic regulators in cells since such quaternary ammonium compounds appear to be much more prevalent in marine organisms than in fresh water animals (18). Indeed, homarine has only been found, thus far, in marine organisms and appears to possess a highly diffusible nature in spite of its highly ionic nature (54). Such an osmo-regulatory function for these compounds would thus attempt to explain the mechanism whereby marine animals maintain a high internal osmotic pressure. Taurobetaine may also be involved in a biosynthetic mechanism leading to choline O-sulfate in the red alga, Gelidium cartilagineum, since this compound is the intermediate postulated by Lindberg (83) for conversion of N,N-dimethyltaurine to choline O-sulfate (see above).

E. Quaternary Ammonium Compounds in Algae: Studies on Amphidinium carteri.

Except for the presence of choline O-sulfate in the red alga Gelidium cartilagineum (83) and laminine in members of the Phaeophyta (121), there have been no non-phospholipid quaternary ammonium compounds identified in algae, a surprising fact in view of the rather widespread occurrence of these compounds in marine organisms. The basic questions thus arise whether such quaternary ammonium compounds do occur to any extent in algae and whether algae, the primary suppliers in the marine food chain, pass such compounds onto higher members of this food chain. Such a production of quaternary amines by algae could thus be likened to the production of toxic substances, such as those produced by the dinoflagellates Gonyaulax catenella and Gymnodinium breve, which are either concentrated in higher marine invertebrates and renders them toxic to higher members of the food chain or

cause massive fish kills (15,90,105). Although the known quaternary amines are not as toxic as such compounds as G. catenella saxitoxin, they may still play an important role in the marine envronment and studies concerned with the occurrence of these compounds in algae, and in particular microscopic algae, could provide insights concerning the roles and movement of such substances in marine organisms.

In 1957, it was reported that supernates containing 1.5×10^6 cells per ml from cultures of the dinoflagellates Amphidinium klebsii and Amphidinium rhynchocephalum caused fish kills in 15-25 minutes (91). Of particular interest was a later report which repeated this initial result with Amphidinium carteri and which led to the postulate that an acetylcholine analog was being released from the alga primarily as a protective agent against zooplankton (129) and secondarily as a fish lethal factor. With this initial indication of a possible biologically-active quaternary ammonium compound in A. carteri and after preliminary tests confirmed the presence of choline in the organism (see below and 72), the present investigation was undertaken. The following study is directed at the extraction, purification and characterization of suspected choline substances in the marine dinoflagellate, Amphidinium carteri.

II. MATERIALS AND METHODS.

A. Organism and Culturing.

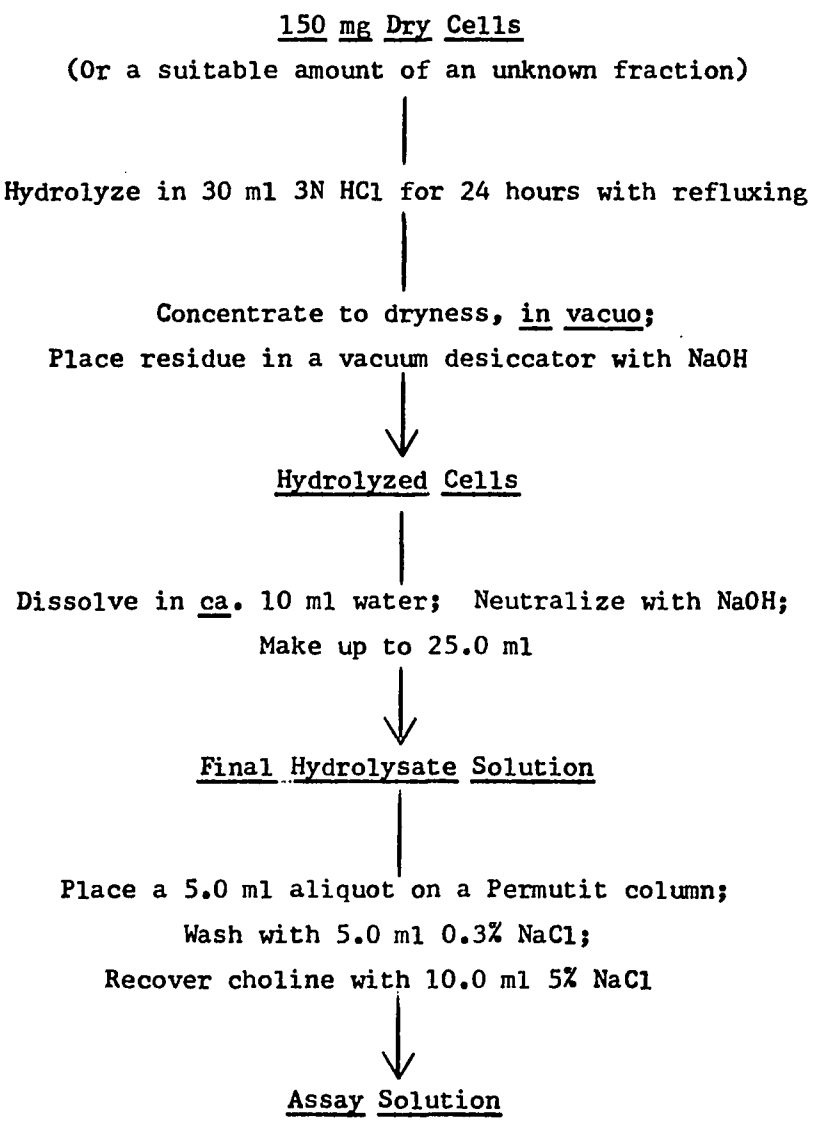
The initial culture of *Amphidinium carteri* utilized in these studies was obtained from Dr. R.L. Guillard (Woods Hole Oceanographic Institute, Woods Hole, Mass.). The dinoflagellate was grown and harvested in the laboratory of Dr. John Sasner, Zoology Department, University of New Hampshire, according to the methods described in APPENDIX I. The initial starter culture of *A. carteri* was obtained bacteria-free and this condition of sterility was maintained throughout its growth period. Frequent microscopic examinations were made using phase contrast microscopy and Gram stains at all stages of the culturing and harvesting in order to determine whether any gross microbial contamination occurred. No such contamination was observed.

B. Neurospora crassa Assay.

SCHEME 1 illustrates the major steps involved in the *Neurospora crassa* assay for choline. The sample to be assayed (either whole dry cells or a specific preparation) was hydrolyzed by refluxing with 30 ml 3N HCl for 18-24 hours. The hydrolysate was concentrated *in vacuo* to dryness and the flask containing the resulting residue was placed in a vacuum desiccator containing a dish of NaOH pellets for 12-24 hours to remove the last traces of acid in the residue. The residue was next dissolved in *ca.* 10 ml distilled water, the resulting solution was neutralized with NaOH and the final volume of the solution was adjusted to 25.0 ml. Aliquots of 5.0 ml of this final hydrolysate were then passed through a Permutit (Fisher Sci-

SCHEME 1.

Neurospora crassa Assay for Choline Activity.



Assay aliquots in Choline Assay Medium (Difco) with N. crassa for 72 hours at room temperature. Collect mycelia and weigh. Compare mycelia weight to a concurrently-run choline chloride standard growth curve.

entific Co., Boston, Mass.) column (100 x 6 mm, containing ca. 1 g Permutit) to separate the choline in the sample from interfering methionine (66). The column was washed with 5.0 ml 0.3% NaCl to remove methionine and then with 10.0 ml 5% NaCl to recover the adsorbed choline from the column. Ten ml of the 5% NaCl eluate was collected and was then assayed for choline with a choline-requiring mutant of N. crassa (strain 485 from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire). Varying amounts of the eluate from 0.1-4.0 ml were added to 125 ml Erlenmeyer flasks, the volume in the flasks was adjusted to 5.0 ml with distilled water and 5.0 ml of double strength Choline Assay Medium (Difco Laboratories, Detroit, Mich.) (66) was added to each flask. The flasks were then sterilized and inoculated with one drop of a suspension of N. crassa spores in distilled water. This suspension was prepared by transfering spores from at least one week old N. crassa slant cultures grown on Malt-Extract Agar (see APPENDIX II) into 5 ml volumes of distilled water until a reading of 80% transmittance on a Bausch and Lomb Spectronic 20 at 650 nm was achieved. The inoculated flasks were then incubated at 25 $\pm$ 2°C for 72 hours after which time the mycelial growth of the N. crassa in the flasks was collected, dried and weighed. The resulting mycelial weights were then related to a concurrently-run choline chloride standard growth curve to determine the amount of choline in the sample. Results are expressed as amount of choline chloride in the unknown sample.

If the sample was to be assayed without prior hydrolysis, it was dissolved in a known volume of 5% NaCl and added directly to the assay flasks. The remainder of the assay was then carried out as described.

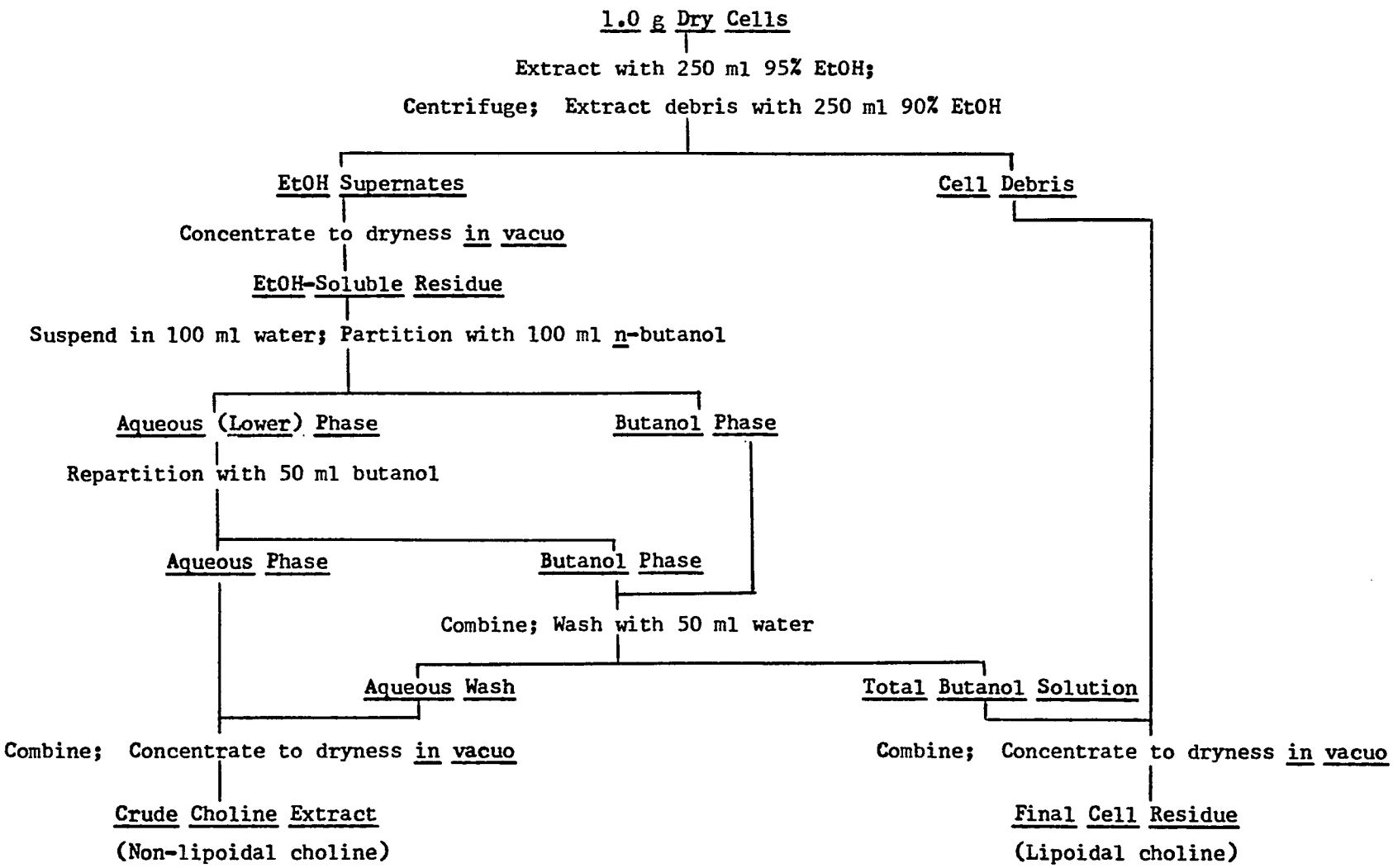
C. Extraction Methods.

1. Extraction of Culture Supernates. The supernate, after centrifuging out the cells at 5000 x g for 10 minutes, of 12 liters of A. carteri culture was concentrated to dryness in vacuo and extracted with 250 ml of hot 95% ethanol. After centrifuging at 45,000 x g to separate the ethanolic phase from insoluble salts, the salts were re-extracted with 250 ml of hot ethanol. The resulting ethanolic extracts were combined and taken to dryness in vacuo and the residue was re-extracted with increasingly smaller volumes of 95% ethanol as well as other alcohols, including propanol, iso-propanol and n-butanol. It was found that such an extraction procedure would not separate the choline substances from the large amounts of salt present in the culture medium and thus this extraction method was abandoned. Other methods involving dialysis, preparative paper chromatography and ion-exchange chromatography were also applied to culture supernates to attempt extraction of the choline substances away from the media salts. These methods also failed and were abandoned.

2. Extraction of Whole Cells (see SCHEME 2). One gram quantities of washed and lyophilized harvested cells of A. carteri were extracted with 250 ml of 95% ethanol for 24 hours on a mechanical stirrer. The resulting ethanolic extract of the cells was centrifuged for 10 minutes at 45,000 x g and the resulting cell debris was next extracted with 250 ml of 90% ethanol. The two ethanolic supernates (which contained any freely-occurring choline compounds as well as most of the lipid-bound choline and pigments of the algal cells) were combined, taken to dryness in vacuo and the resulting residue was suspended in 100 ml distilled water and partitioned against 100 ml n-butanol in a separatory funnel. After standing for ca.

SCHEME 2.

Isolation of Choline Compounds from Amphidinium carteri.



2 hours, the clear, slightly-yellow lower aqueous phase of the partition mixture was drawn off and repartitioned with 50 ml of fresh n-butanol. The two upper phase butanol solutions resulting from these partitions were combined and washed with 50 ml of distilled water to ensure that all the water-soluble components of the extract were removed from this butanol solution. The butanol solution was then combined with the cell debris from the ethanolic extraction of the whole cells and this mixture was taken to dryness in vacuo and saved for further analysis (Final Cell Residue, see SCHEME 2). The water wash of the butanol solution was combined with the aqueous partition phase, the combined solution was taken to dryness in vacuo, and the resulting residue was analyzed as described below. Hereafter, this aqueous partition fraction residue of the ethanolic extract of whole A. carteri cells will be referred to as the Crude Choline Extract.

D. Paper and Thin-Layer Chromatography.

1. Standard Samples. Standard samples (50 mg/ml in 50% ethanol) used included choline chloride (Fisher Scientific Co., Boston, Mass.); acetyl-propionyl- and butyrylcholine chloride (Sigma Chemical Co., St. Louis, Mo.); succinyldicholine dichloride (Sigma); acetyl-β-methylcholine bromide (Sigma); choline O-phosphate (Sigma); choline O-sulfate (Professor A.A. Benson, Scripps Institution of Oceanography, La Jolla, Calif.); ethanolamine (Eastman Kodak Co., Rochester, N.Y.); N-methylethanolamine (K & K Laboratories, Inc., Plainview, N.Y.); and N,N-dimethylethanolamine (Sigma). Acrylylcholine was synthesized by the modification of a reported method (107) in the laboratory of Dr. Kenneth Andersen, Department of Chemistry, University of New Hampshire (see APPENDIX III).

2. Paper Chromatography. Whatman No. 1 paper (35 x 20 cm) was used in all cases. The paper was spotted with ca. 10 ul of known and unknown samples along the longer dimension of the paper, rolled into a cylinder, stapled, and placed into a covered, cylindrical glass tank at 22-25°C. The chromatogram was then run ascendingly until the solvent front was within 1-2 cm of the top of the paper. After drying, the choline compounds were located first by exposing the chromatograms to iodine vapors which detected all the standards with the occasional exception of choline O-sulfate and choline O-phosphate. After the iodine treatment, the chromatograms were sprayed with Dragendorff's Reagent (see below) for positive identification of choline compounds.

The solvent systems used for paper chromatography are listed in TABLE 3. Besides using Whatman No. 1 paper per se, it was also used after modification by treatment with potassium chloride. This treatment involved drawing the correctly-sized paper through a solution of 1M KCl and allowing the paper to dry before use (26,27). The solvent to be used with the treated paper was also modified by saturating it with solid KCl in the chromatography tank for at least one hour before the chromatogram was run.

R_f values for the standard and known samples in paper and thin-layer systems were calculated as:

$$R_f = \frac{\text{Distance from origin to spot center}}{\text{Distance from origin to solvent front}}$$

3. Thin-Layer Chromatography (TLC). Prepared 20 x 20 cm, 0.25 mm thick silica gel (E. Merck) and alumina (Macherey, Nagel) pre-coated glass plates (Brinkman Instruments, Inc., Westbury, N.Y.) were used in all cases. The plates were stored in a desiccator until their immediate use and were

TABLE 3.

Paper Chromatographic Solvent Systems

Number	Solvent System (v/v)	References
1	Ethanol:acetic acid:water (90:5:5)[a]	55
2	Ethanol:NH_4OH:water (90:5:5)[a]	55
3	n-Propanol:formic acid:water (8:1:1)[a]	139
4	t-Butanol:acetic acid:water (70:15:15)[a]	
5	n-Butanol:acetic acid:water (4:5:1)	80
6	t-Butanol:formic acid:water (70:15:15)	37
7	n-Propanol:water (7:3)	37
8	Acetone:water (9:1)	60
9	Acetone:water (9:1)[a]	
10	n-Propanol:acetic acid:water (8:1:1)[a]	
11	Acetone:acetic acid:water (8:1:1)[a]	
12	Phenol:n-butanol:water:formic acid (50:50:10:3)	26
13	n-Propanol:formic acid:water (6:3:1)	27
14	n-Butanol:pyridine:water (6:4:3)	29
15	t-Butanol:pyridine:water (6:4:3)	
16	n-Propanol:pyridine:water (6:4:3)	
17	2-Propanol:pyridine:water (6:4:3)	
18	2-Propanol:pyridine:water (6:4:3)[a]	
19	2-Propanol:acetic acid:water (8:1:1)	
20	2-Propanol:acetic acid:water (8:1:1)[a]	
21	2-Propanol:formic acid:water (8:1:1)	
22	2-Propanol:formic acid:water (8:1:1)[a]	

TABLE 3 (Continued).

Number	Solvent System (v/v)	References
23	n-Propanol:water (9:1)[a]	139
24	n-Propanol:1M acetic acid (3:1)[a]	
25	n-Butanol:ethanol:acetic acid:water (8:2:1:3)	12, 77
26	n-Propanol:1M acetic acid (3:1)	77
27	n-Butanol:saturated with water	14, 77, 139
28	n-Butanol:propanol:water (60:30:15)	14, 139

[a]System used with KCl paper and KCl-saturated solvent.

used without any prior activation at elevated temperatures. The plates were spotted with ca. 5 ul standard and unknown samples and were placed in covered rectangular glass TLC chambers which had been lined with Whatman 3MM paper and equilibrated with the solvent to be used for at least one hour prior to the run. After the solvent had ascended to within 1-2 cm of the top of the plate, the plate was removed, dried, and subjected to iodine vapors and Dragendorff's Reagent as described for paper chromatography. In addition, silica gel plates were sprayed with 50% sulfuric acid and charred as a further check of the location of organic material on the plates.

The solvent systems and specific plates used in thin-layer chromatography are described in TABLE 4.

4. Chromatographic Detection Methods. Detection of the choline compounds and ethanolamines on paper and thin-layer chromatograms utilized a number of specific and non-specific reagents.

a. Iodine vapors. Iodine crystals were placed in a large desiccator jar and the jar was allowed to fill with iodine vapors. Choline compounds and ethanolamines developed as orange-brown spots on chromatograms placed in the vapor-filled jar.

b. Dragendorff's Reagent (Modified after 115). The following stock solutions were stored at 4°C:

(1) 1.7 g bismuth nitrate in 10 ml 20% acetic acid

(2) 4.0 g potassium iodide in 10 ml distilled water

Immediately before use, 1.0 ml of each of the two stock solutions were added to 2.0 ml glacial acetic acid in 10.0 ml distilled water in a spray bottle. The spray was used immediately on the chromatograms and the

TABLE 4.

Thin-Layer Chromatography Systems

Number	Solvent System (v/v)	Support	References
1	n-Butanol:water:formic acid (60:35:15)	alumina	115
2	n-Butanol:water:acetic acid (60:17:17)	alumina	115
3	n-Butanol:water:acetic acid (4:5:1)	alumina	115
4	Methanol:CCl_4:acetic acid (28:12:1)	alumina	120
5	Methanol:HCl (95:5)	alumina	101
6	Ethanol:7% ammonia (2:1)	alumina	101
7	n-Butanol:water:acetic acid (4:1:1)	alumina	122
8	Phenol:t-butanol:formic acid:water (50:50:3:10)	alumina	
9	n-Propanol:phenol:water:acetic acid (4:4:1:1)	silica gel	
10	n-Propanol:pyridine:water:acetic acid (6:4:3:1)	silica gel	
11	Phenol:2-propanol:formic acid:water (50:50:3:10)	silica gel	
12	Ethanol:water:acetic acid (4:1:1)	silica gel	

sprayed chromatograms were placed in a 100° C oven for 5 minutes to dry. This reagent is specific for choline and other quaternary ammonium bases as well as for some alkaloids (113,115). Choline compounds appear as yellow to light orange (choline O-sulfate and choline O-phosphate), orange to orange/blue (choline esters), or orange-pink (choline chloride) spots on sprayed chromatograms while the ethanolamines do not respond to this reagent.

c. Hanes-Isherwood Reagent (59). The following stock solutions were stored at 4° C:

(1) 4% (w/v) $(NH_4)_2MoO_4$ in distilled water

(2) 1N HCl

(3) 60% (v/v) $HClO_4$ in distilled water

Immediately before use, the solutions were mixed in the ratio 25:10:5 and sprayed onto the dried chromatograms. The reagent is specific for phosphate esters (115) and was used to ascertain the presence or absence of choline O-phosphate in extracts of A. carteri. Esters of phosphate, including choline O-phosphate, develop as blue spots on the sprayed chromatograms after exposure to UV light.

d. Charring with 50% sulfuric acid. Silica gel thin-layer plates were sprayed with 50% sulfuric acid in distilled water (v/v) and placed in an oven at 125-150° C for 2-6 hours. Organic matter on the plates developed as black spots.

E. Initial Purification of the Choline Compounds from A. carteri.

1. Column Chromatography. Rexyn 102 (H^+)(Fisher Scientific Co., Boston, Mass.) and AG 50W-X4 (Bio-Rad Laboratories, Richmond, Calif.)

cation exchange resins were used in attempts to purify the Crude Choline Extract. The column packing to be used was washed in 1N HCl and packed in a 2 cm diameter glass column to give a 15 cm resin bed. After running ca. 500 ml of 1N HCl through the packed column to assure the resin was in its active form, the column was washed to neutrality with ca. 1000 ml distilled water. The Crude Choline Extract resulting from the extraction of 1.0 g of A. carteri whole cells was dissolved in water and applied to the column at pH 7. Elution was carried out with 500 ml volumes of water and then increasingly more acidic solutions from 0.01M acetic acid to 1N HCl. Fractions of 50 ml were collected and subjected to paper chromatographic analysis to locate the choline compounds.

2. Preparative Paper Chromatography. Preparative paper chromatography of the unknown choline compounds from A. carteri was run on 40 x 50 cm sheets of Whatman No. 1 paper in both KCl and non-KCl systems. The Crude Choline Extract from the ethanolic extraction of ca. 0.5 g of A. carteri whole cells (see SCHEME 2) was spotted onto the paper and the chromatogram was run in a covered glass tank with 400 ml of an appropriate solvent for 20 hours. After the run, a thin vertical strip was cut from the chromatogram and sprayed with Dragendoroff's Reagent to locate the choline compounds. The strip was then realigned with the chromatogram and the chromatogram was sectioned to separate the areas corresponding to the Dragendorff-positive spots on the developed strip. The sectioned chromatogram was then cut apart and each section was cut into pieces and eluted with distilled water. The resulting solutions were separated from the chromatogram paper, taken to dryness in vacuo, and the residues stored in the deep freeze for further analysis and purification.

In most cases, preparative chromatography utilizing this method was run in paper chromatography systems 1 or 17 (see TABLE 3). The resulting Dragendorff-positive material recovered from sectioned chromatograms was then either subjected to physiological and N. crassa assays or used for further purification and analysis (see below).

F. Physical, Chemical and Spectral Methods.

1. Melting Points. Melting points of purified choline compounds were determined on a Thomas-Hoover Capillary Melting Point Apparatus. When possible, a second capillary containing the known standard suspected to be identical with the unknown sample was run concurrently with the unknown sample.

2. Hydroxylamine-Ferric Chloride Reaction (64). This reaction is used for the colorimetric determination of short chain carboxylic acid esters, lactones and aldehydes. Approximately 0.1 mg of the unknown sample in 1 ml water is added to 1 ml of 2M hydroxylamine-HCl and 1 ml of 3.5N NaOH. After two minutes, 1 ml of 33% (v/v) HCl in water and 1 ml of $FeCl_3$ solution (0.37M $FeCl_3$ in 0.1N HCl) are added to the reaction mixture. The resulting purple color given by esters, lactones and aldehydes can be used as a quantitative colorimetric or qualitative determination and has been applied to the identification and estimation of choline esters (64).

3. Hydrolysis and Subsequent Extraction of Choline Esters. The unknown or standard samples (5-10 mg) were dissolved in 5 ml of 3N HCl and refluxed for 4-6 hours. After cooling, two procedures were followed depending on whether recovered choline chloride or the derived acid from the ester was to be determined. If choline chloride was to be determined, the

hydrolysate was taken to dryness *in vacuo* and placed in a vacuum desiccator with NaOH pellets to remove all traces of HCl. The resulting residue was then used for the determination of choline chloride by either the *N. crassa* assay or paper chromatography. If the derived acid of the hydrolyzed ester was to be determined, the hydrolysate was extracted with an equal volume of diethyl ether three times. The resulting ether extracts were then combined, taken to a minimal volume (*ca.* 0.1 ml) *in vacuo* at room temperature, and the resulting material was analyzed by gas-liquid chromatographic and spectral methods.

4. Gas-Liquid Chromatography (GLC). A Barber-Colman Model 5000 Gas Chromatograph equipped with a hydrogen flame ionization detection system was used. Known standards and the acids derived from hydrolyzed choline esters were analyzed on a 6 ft x 4 mm (i.d.) glass U-tube column packed with 100-120 mesh Porapak Q (Waters Associates, Inc., Framingham, Mass.). The column was conditioned for 8 hours at 250°C with a nitrogen carrier gas flow rate of 45 ml/min. Blocking of active sites on the packed column was accomplished by periodic injections of SILYL-8 (Pierce Chemical Co., Rockford, Ill.). Analysis of both known and unknown samples were run isothermally at 200, 225 and 240°C with a carrier gas flow rate of 40 ml/min. Detector and injector temperatures were routinely maintained at 25°C above the column temperature except in the case when the column was run at 200°C. In the latter case, the detector was maintained at 250°C.

Standard and unknown samples (1-5 ul of 10-20 mg/ml solutions in glass-redistilled methanol) were injected directly onto siliconized glass wool plugs in the column. Acetic, propionic, butyric, *iso*-butyric, chloracetic, glycolic and lactic acids; ethyl-, propyl-, *iso*-propyl-, butyl-, *iso*-butyl-,

t-butyl-, n-amyl-, iso-amyl- and t-amyl alcohols; acetone and methyl ethyl ketone were obtained from Fisher Scientific Co., Boston, Mass. Valeric, α -methylbutyric, methoxyacetic and iso-valeric acids and sec-butyl alcohol were obtained from Eastman Kodak Co., Rochester, N.Y. Caproic, crotonic, β ,β-dimethylacrylic, glyoxylic and glyceric acids were obtained from Sigma Chemical Co., St. Louis, Mo. Butyryl-, acrylic, crotonic and glycid-aldehyde; β -propiolactone and γ-butyrolactone were obtained from Aldrich Chemical Co., Milwaukee, Wis. Acrylic acid was obtained from the Baker Chemical Co., New York, N.Y., and pyruvic acid from Calbiochem, Los Angeles, Calif. If necessary, the acids and alcohols were glass-redistilled to a constant boiling point before GLC analysis.

Retention times (t) were calculated from the point of injection to the midpoint of the eluted chromatogram peak of the compound. Relative retention times (R) for specific compounds were calculated by the relationship:

$$R = \frac{t \text{ of the specific compound}}{t \text{ of the series standard}}$$

In the case of the alcohols, the series standard was ethanol. The other compounds, acids, aldehydes, etc., were compared to acetic acid as the series standard. The relative retention time of the series standard was set equal to 1.00.

5. Spectral Analysis. Ultraviolet/visible spectra were determined in absolute methanol or ethanol on a Cary Model 15 recording spectrophotometer scanning from 210-800 nm or on a Coleman 124 Double Beam Grating Spectrophotometer (Perkin-Elmer Corp.) scanning from 350-190 nm. Infrared spectra were determined in absolute ethanol on a Perkin-Elmer Model 700 Infrared

Spectrophotometer.

G. Toxicity Tests.

1. Initial Testing. Initial toxicity tests of A. carteri cultures and fractions were carried out with killifish (Fundulus heteroclitus), guppies (Lebestes reticulatus) and white mice in the laboratory of Dr. John Sasner, Zoology Department, University of New Hampshire. Whole cultures, culture supernates and whole cells of A. carteri were assayed in these tests.

2. Toxicity of Choline Standards in Mice. In order to determine the approximate toxicities of choline compounds in mice, known amounts of standard choline compounds were dissolved in distilled water and 0.25 ml of solution was injected intraperitoneally into 19-21 g male and virgin female white mice. Concentrations of the known compounds were then adjusted to determine the approximate ($\pm$ 10%) minimum lethal dose (MLD) for each compound, which is defined as that amount of the compound in milligrams which, when dissolved in 0.25 ml water and injected IP into 19-21 g mice, causes death within 24 hours. The MLD for each compound once estimated was confirmed in at least 5 male and 5 female mice.

3. Toxicity of Choline Esters in Fish. In order to determine the effect of choline esters in sea water, choline esters were tested for their possible activity on Fundulus heteroclitus. Killifish (4-6 cm body length) were collected in Great Bay Estuary, Newington, New Hampshire, and placed in Seven Seas Marine Mix (artificial seawater)(Utility Chemical Co., Patterson, N.J.) for at least 24 hours before testing. Known amounts of the compounds to be tested for toxicity were dissolved in 500 ml volumes of artificial seawater to result in a series of concentrations for each com-

pound from 0.5-10 mg/ml. Initially, two fish were tested at each concentration level until a lethal level was found. Once the lethal level for a compound was found, at least five more fish were used to confirm the level. If no death occurred at a concentration of 10 mg/ml for a compound, it was considered non-toxic.

H. Pharmacological Studies.

The crude and purified choline components from A. carteri were tested in a number of physiological systems in the laboratory of Dr. John Sasner, Department of Zoology, University of New Hampshire, according to methods already described in detail (104, 125). Isolated and in vivo heart preparations of Mercenaria (Venus) mercenaria, in vivo heart assays on Cancer irroratus and Carcinus maenus, intracardiac injection of Rana pipiens heart, and the contraction of mouse intestine were utilized in these tests. Results of unknowns in these systems were compared to the activity of concurrently-run choline standards.

III. RESULTS AND DISCUSSION.

A. Initial Observations on Toxicity.

The first indication of the presence of toxic factors in Amphidinium carteri was observed when killifish placed in beakers containing 400 ml of supernate from a centrifuged culture of A. carteri in late stationary phase died within 30-50 minutes. Further evidence of toxicity was found when guppies placed in an aquarium containing 0.05 mg/ml of whole A. carteri cells died in 20-30 minutes. Controls, when placed in aged culture medium or in an aquarium containing 0.05 mg/ml of whole cells of Spirogira sp., were not affected. In addition, when 25 mg of whole A. carteri cells was injected IP into white mice, death occurred in 80-140 minutes. The same amount of Spirogira sp. cells caused no death in control mice.

These initial toxicity indications along with the theory that toxic factor(s) of A. carteri might be choline-like in nature (91,129) led us to assay whole dry cells of the organism for choline, utilizing the N. crassa assay. The results of this assay revealed that A. carteri contains from 0.36-0.41% dry weight of choline chloride equivalent. Thus, further attempts were made to extract and characterize the choline compounds produced by this alga.

B. Neurospora crassa Assay.

The N. crassa assay was initially tested with known standards to determine its specificity with respect to choline derivatives. It was found to respond equally well, on a molar basis, to choline, choline O-sulfate, N,N-dimethylethanolamine and N-methylethanolamine; to a lesser extent to

succinyldicholine, choline O-phosphate, acetyl-, propionyl- and butyrylcholine; and not significantly to acetyl-β-methylcholine and ethanolamine (see FIGURES 1 and 2). In addition, other workers have found no response is elicited in the assay by betaine, creatine, sarcosine, neurine, N,N-diethylethanolamine, dimethylamine, trimethylamine and tetramethylammonium chloride (57). Thus, it appears that the assay is specific for choline derivatives, the only exceptions being N-methyl- and N,N-dimethylethanolamine. In addition, the assay appears to show a graded response of decreasing mycelial growth as choline is esterified and as the chain length of the acyl portion of the ester becomes longer.

C. Extraction of Choline Compounds from A. carteri.

Initial attempts at isolating the choline factors from A. carteri were aimed at recovering the compounds from culture supernates since the supernate from late growth phase cultures caused fish kills. However, the low molecular weight, dialyzable nature of the choline factors together with the high solubility of the factors in aqueous and ethanolic solutions made extraction of the factors away from the large amounts of salts in the medium unsuccessful. Thus, the factors were extracted from harvested, salt-free whole cells of A. carteri in early stationary phase of growth under the assumption that any non-lipoidal unbound choline derivatives are initially inside the whole cells at early stationary growth phase and are released by the cells into the medium in late stationary phase where they may act as toxic factors. This assumption is supported, in part, by the fish toxicity reactions to late culture supernates and to whole dry cells as reported above. Thus, the ethanolic extraction procedure of whole cells (SCHEME 2) was used to extract non-lipoidal choline

FIGURE 1. Mg mycelium of N. crassa resulting from the addition of increasing concentrations of choline esters to choline-free media. 1, Choline chloride; 2, succinyldicholine dichloride; 3, acetylcholine chloride; 4, propionylcholine chloride; 5, butyrylcholine chloride; 6, acetyl- β-methylcholine bromide.

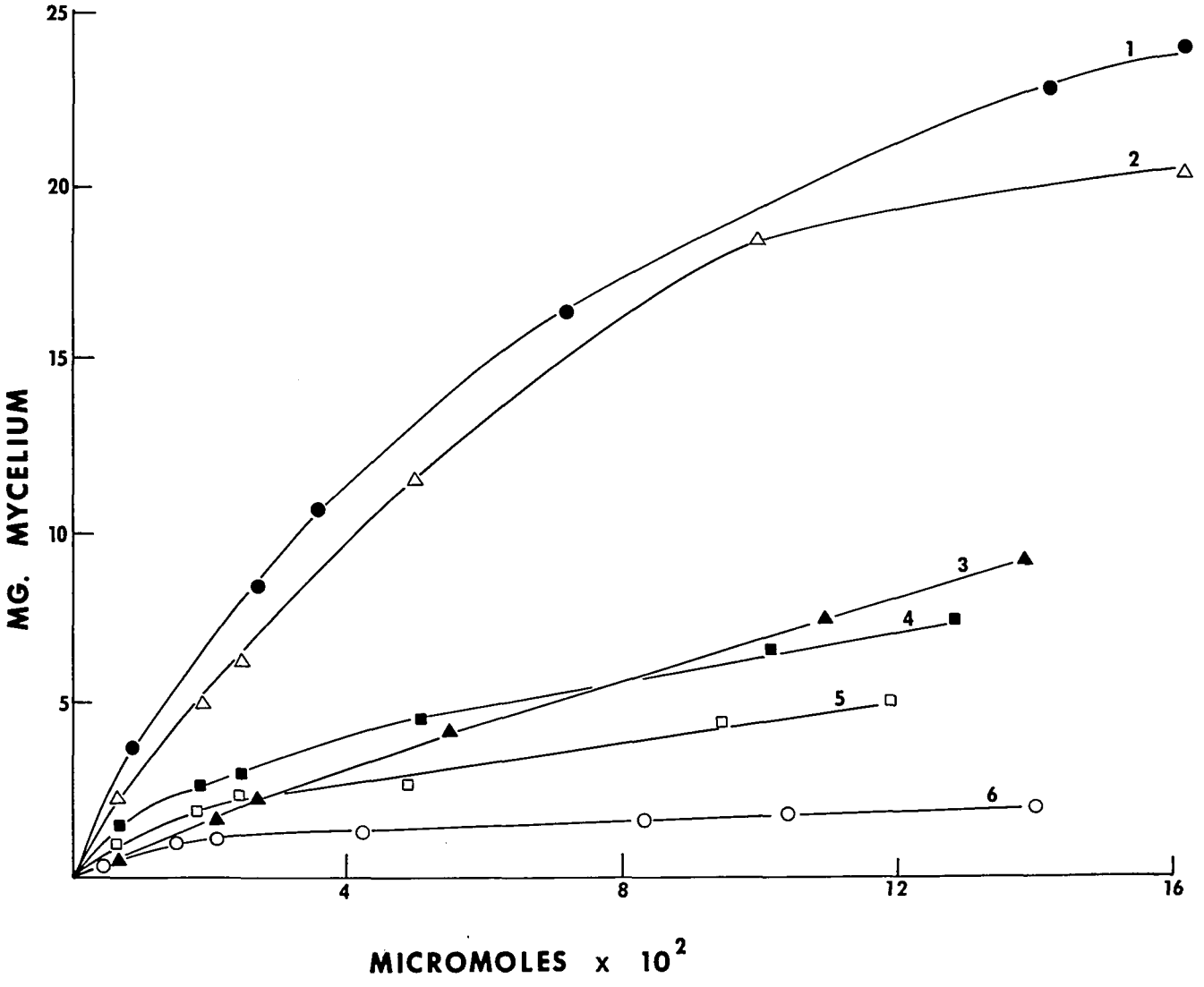
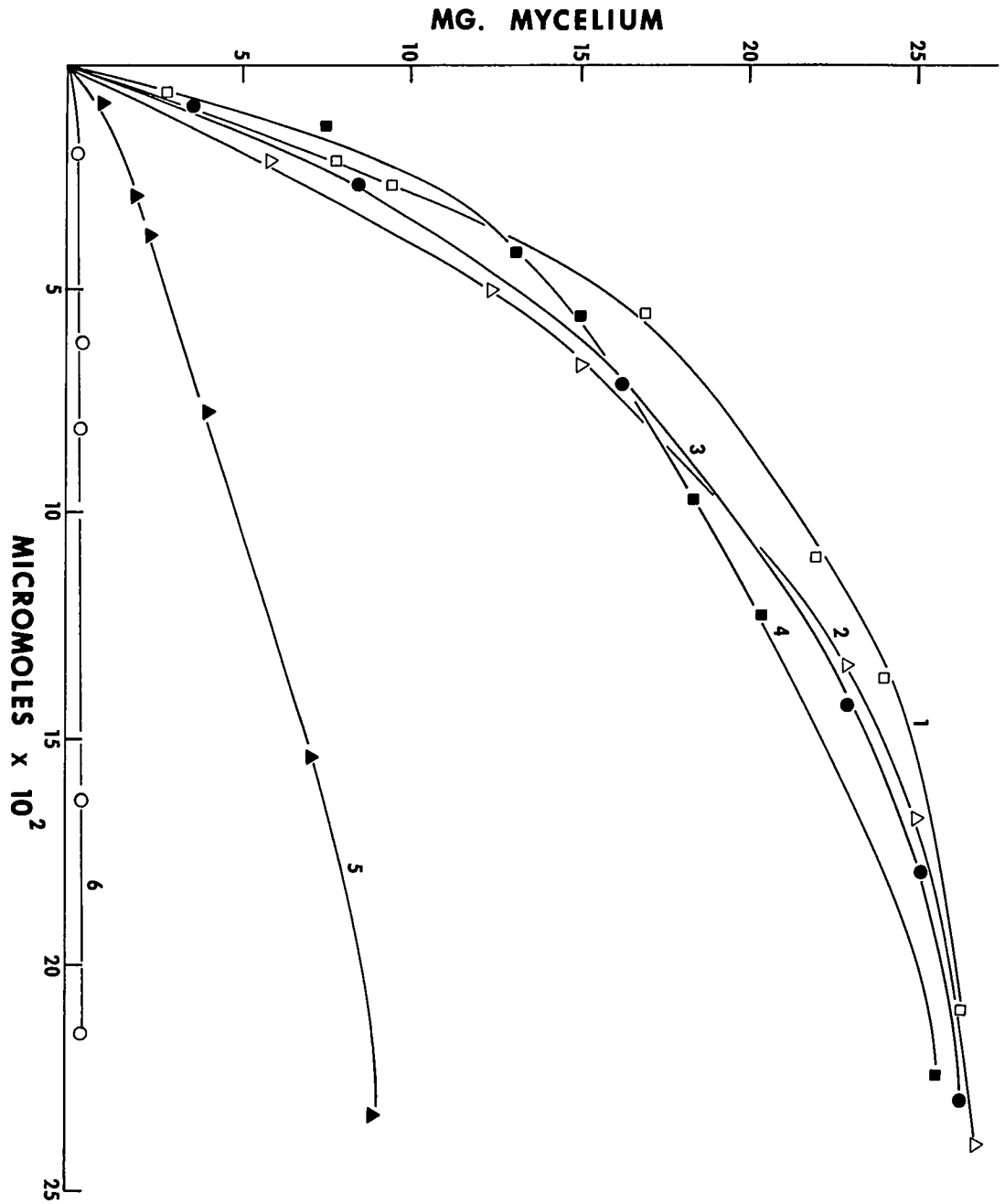

25
20
15
10
5
MG. MYCELIUM
1
2
3
4
5
6
4
8
12
16
MICROMOLES x 10^2

FIGURE 2. Mg mycelium of N. crassa resulting from the addition of increasing concentrations of choline derivatives and ethanolamines to choline-free media. 1, Choline O-sulfate; 2, N-methylethanolamine; 3, choline chloride; 4, N,N-dimethylethanolamine; 5, choline O-phosphate; 6, ethanolamine.


MG. MYCELIUM
5
10
15
20
25
MICROMOLES x 10^2
5
10
15
20
25
1
2
3
4
5
6

compounds from A. carteri.

D. Estimation of Lipoidal and Non-Lipoidal Choline in A. carteri.

The Neurospora assay as described above estimates the total choline content of whole cells since the sample is acid hydrolyzed prior to assay. Thus, choline bound in non-pharmacologically active forms such as in phospholipids is released from these compounds during hydrolysis and estimated as free choline along with choline from pharmacologically active derivatives. To estimate the amount of free and pharmacologically active non-lipoidal choline present in A. carteri, the Crude Choline Extract from the ethanolic extraction proceedure (SCHEME 2) was assayed, unhydrolyzed, in the N. crassa assay. In addition, the cell debris/butanol combined fraction of the extracted cells (Final Cell Residue, SCHEME 2) was assayed both before and after acid hydrolysis. The results of these assays are shown in SCHEME 3. Water-insoluble and lipoidal forms of choline account for only 0.07-0.10% of the dry weight of the original A. carteri cells while the water-soluble non-lipoidal forms of choline comprise most of the choline in the organism and elicit a positive Neurospora response without prior hydrolysis. The unhydrolyzed cell debris/butanol fraction elicited no response in the assay.

It is thus concluded from these results that the major forms of choline occurring in A. carteri are non-lipoidal in nature.

E. Paper and Thin-Layer Chromatographic Analysis.

1. Standard Samples. All R_f values determined in this investigation are averages of at least five chromatographic runs for each sample. Any individual run for any standard did not deviate from the average R_f by more than 1%. TABLES 5 and 6 report the R_f values for known choline standards in 28 paper chromatographic systems. While TABLE 5 reports the actual R_f values determined in the present investigation, TABLE 6 is a compilation of both R_f values de-

SCHEME 3.

Correlation of Non-Lipoidal and Lipoidal Choline in Amphidinium carteri.

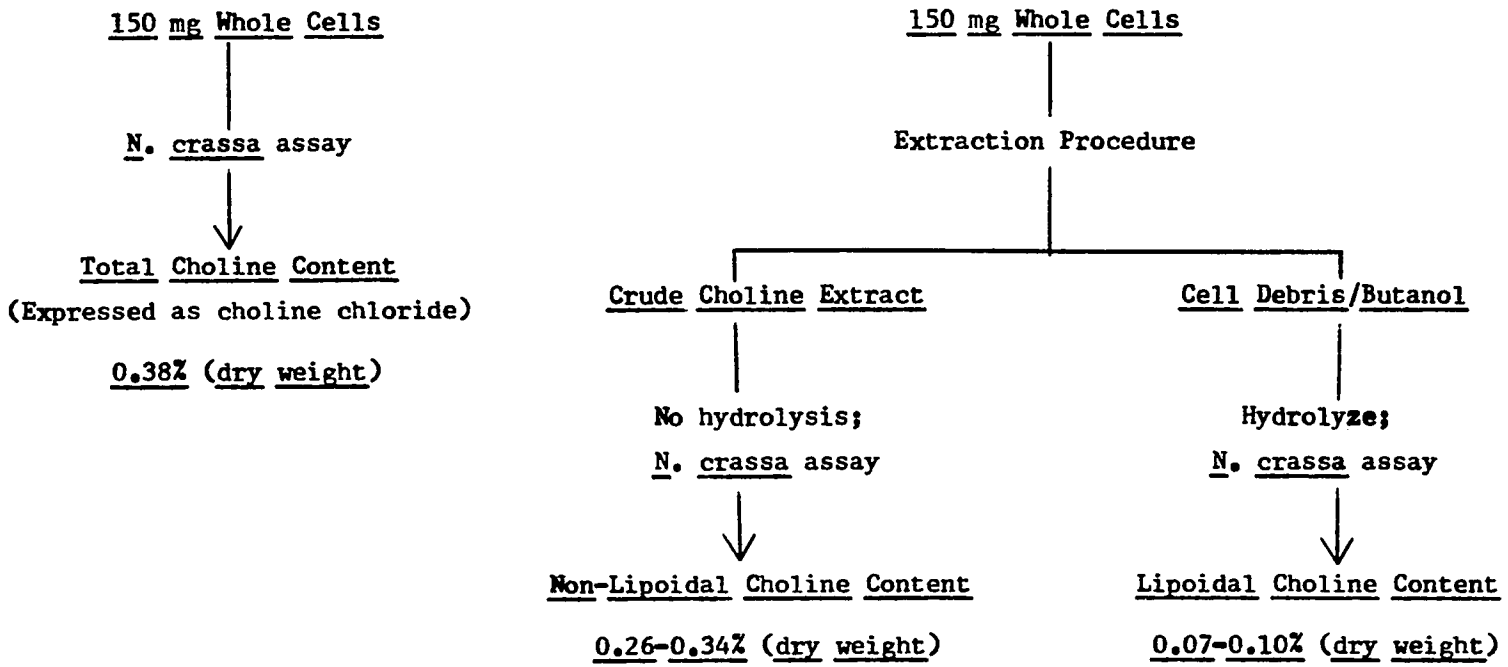


TABLE 5.

R_f Values of Choline and Related Compounds on Paper Chromatography.

Compound	R_f Value in System[a,b]:											
	1	2	3	4	5	6	7	8	9	10	11	12
Choline chloride	0.52	0.60	0.47	0.25	0.30	0.57	0.52	0.17	0.16	0.41	0.27	0.61
Acetylcholine chloride	0.60	0.63	0.57	0.33	0.37	0.64	0.57	0.25	0.30	0.49	0.47	0.72
Acetyl-β-methylcholine bromide	0.71	0.74	0.66	0.48	0.47	0.69	0.68	0.54	0.45	0.60	0.61	0.77
Propionylcholine chloride	0.68	0.70	0.66	0.48	0.48	0.73	0.65	0.38	0.42	0.59	0.59	0.76
Butyrylcholine chloride	0.75	0.77	0.73	0.57	0.56	0.78	0.72	0.46	0.55	0.67	0.69	0.79
Succinyldicholine dichloride	0.25	0.54	0.21	0.06	0.15	0.34	0.38	0.04	0.03	0.11	0.07	0.60t
Choline O-phosphate	0.09	0	0.07	0.02	0.17	0.17	0.06	0	0	0.03	0	0.48
Choline O-sulfate	0.14	0.21	0.12	0.06	0.18	0.35	0.36	0.12	0.07	0.07	0.12	0.58
Ethanolamine	0.47	0.64	0.36	0.40	0.39	0.57	0.71	0.79	0.66	0.39t	0.40	0.30
N-Methylethanolamine	0.56	0.77	0.45	0.41	0.43	0.62	0.79	0.80	0.86	0.43t	0.43	0.47
N,N-Dimethylethanolamine	0.60	0.81	0.48	0.37	0.40	0.64	0.82	0.82	0.84	0.43t	0.41	0.63

[a] See TABLE 3 for solvent systems.

[b] Abbreviations: t = tailing.

TABLE 5 (Continued)

Compound	R_f Value in System[a,b]:											
	13	14	15	16	17	18	19	20	21	22	23	24
Choline chloride	0.74	0.28	0.47	0.48	0.52	0.43	0.40	0.33	0.51	0.48	0.30	0.38
Acetylcholine chloride	0.80	0.32	0.54	0.49	0.55	0.48	0.46	0.41	0.59	0.57	0.37	0.46
Acetyl-β-methylcholine bromide	0.88	0.51	0.67	0.61	0.67	0.61	0.58	0.53	0.68	0.69	0.52	0.57
Propionylcholine chloride	0.87	0.44	0.62	0.58	0.63	0.60	0.56	0.53	0.67	0.67	0.51	0.59
Butyrylcholine chloride	0.91	0.51	0.69	0.65	0.69	0.69	0.65	0.60	0.72	0.76	0.62	0.70
Succinyldicholine dichloride	0.72	0.10	0.22	0.18	0.26	0.15	0.13	0.07	0.27	0.19	0.05	0.27
Choline O-phosphate	0.55	0	0	0	0	0	0.05	0	0.13	0.06	0	0.04
Choline O-sulfate	0.60	0.20	0.37	0.39	0.41	0.17	0.13	0.05	0.18	0.12	0.03	0.12
Ethanolamine	0.63	0.40	0.63	0.53	0.60	0.50	0.44	0.36	0.45	0.38t	0.52t	0.34
N-Methylethanolamine	0.73	0.59	0.71	0.69	0.73	0.64	0.53	0.41	0.55	0.43t	0.60t	0.39
N,N-Dimethylethanolamine	0.79	0.77	0.78	0.85	0.86	0.74	0.52	0.40	0.60	0.48t	0.61t	0.35

[a] See TABLE 3 for solvent systems.

[b] Abbreviations: t = tailing.

TABLE 6.

Compiled R_f Values of Choline and Related Compounds on Four Paper Chromatographic Systems.

Compound	R_f Value in System[a,b]:			
	25	26	27	28
Choline chloride	0.37	0.46	0.09	0.15
Acetylcholine chloride	0.46	0.54	0.14	0.21
Acetyl- -methylcholine bromide	0.54	0.61	0.22	0.30
Propionylcholine chloride	0.57	0.61	0.22	0.31
n-Butyrylcholine chloride	0.66	0.69	0.28	0.40
2-Methylbutyrylcholine bromide	0.73	0.80	0.36	----
n-Valerylcholine bromide	0.69	0.78	0.31	0.55
iso-Valerylcholine chloride	0.78	0.82	0.38	----
n-Caproylcholine bromide	0.68	0.79	0.36	----
Succinyldicholine dichloride	0.22	0.29	0	0.02
Benzoylcholine chloride	0.71	0.69	0.28	0.30
Crotonylcholine bromide	0.50	0.71	0.20	----
n-Pentenoylcholine bromide	0.71	0.71	0.35	----
β,β-Dimethylacrylylcholine bromide	0.70	0.71	0.35	----
Urocanylcholine bromide	----	0.54	0.06	----
Imidazolylpropionylcholine iodide	0.37	0.29	0.15	----
Indolylacetylcholine bromide	0.63t	0.73t	0.2 t	----
Indolylpropionylcholine bromide	0.60	0.73	0.26	----
Nicotinoylcholine perchlorate	0.35	0.58	0.10	----
Betaine	0.46	----	----	----
Carbaminoylcholine chloride	0.30	----	----	----
Choline O-sulfate	0.31	0.32	0	0.03
Choline O-phosphate	0.10	0.15	0	0
Ethanolamine	0.45	0.52	0.34	0.46
N-Methylethanolamine	0.50	0.56	0.59	0.57
N,N-Dimethylethanolamine	0.48	0.53	0.81	0.66

[a]See TABLE 3 for solvent systems and references.

[b]Abbreviations: t = tailing.

termined in this investigation and the R_f values of choline compounds reported by other workers. Since many of the compounds in TABLE 6 were not available for testing and involve the work of different investigators, the R_f values of the substances in this table may be expected to vary (ca. $\pm$ 10%) due to the slightly different conditions used for their determinations.

The solvent systems found to work best in paper chromatography almost always contained an alcohol and an acid as well as always containing water. However, there were systems employing pyridine or ammonium hydroxide which worked equally as well as the acidic systems, contrary to the reports of some workers that bases such as ammonium hydroxide will cause hydrolysis of choline esters on the chromatograms (12,139). The use of KCl-treated paper and solvent generally resulted in less tailing and more compact spots as well as usually lower R_f values for samples than the same system without treated paper and solvent. However, in some cases, the KCl-treated paper was undesirable due to the appearance of two solvent fronts and, in other cases, equally good results were obtained in the same system with either treated or untreated paper.

TABLE 7 reports the R_f values of known choline standards in 12 thin-layer chromatographic systems. Solvent systems for these chromatograms were more varied in nature than paper chromatographic systems, but, again, the systems were primarily based on an alcohol, acid and water.

In both paper and thin-layer chromatographic analysis, it was observed that in almost all systems, an increase in the chain length of a choline ester by one carbon unit resulted in increased R_f values and, in the case of paper chromatography, this increase was fairly consistant at 0.05-0.10 R_f units in agreement with other workers (14). Also in agreement with other workers, isomeric esters such as propionyl- and acetyl-β-methylcholine had very similar R_f values in both paper and thin-layer systems (139), and succinyldicholine had the lowest R_f value of all the choline esters in all systems

TABLE 7.

R_f Values of Choline and Related Compounds on Thin-Layer Chromatography.

Compound	R_f Value in System[a,b]:											
	1[c]	2	3[c]	4[c]	5[c]	6	7[c]	8	9	10	11	12
Choline chloride	0.47	0.51	0.45	0.55	0.55	0.73	0.76	0.65	0.46	0.27	0.48	0.24
Acetylcholine chloride	0.53	0.56	0.52	0.62	0.63	0.78	0.85	0.76	0.53	0.31	0.55	0.26
Acetyl-β-methylcholine bromide	0.59	0.69	0.73	0.76	0.75	0.84	0.93	0.82	0.61	0.35	0.62	0.29
Propionylcholine chloride	0.55	0.61	0.56	0.70	0.68	0.79	0.89	0.80	0.57	0.36	0.59	0.31
Butyrylcholine chloride	0.60	0.63	0.62	0.69	0.67	0.81	0.92	0.81	0.62	0.38	0.63	0.35
Succinyldicholine dichloride	0.18	0.58	0.31	0.56	0.64	0.80	0.77	0.72	0.31t	0.10	0.36	0.11
Choline O-phosphate	0.07	0.03	0.06	0	0	0	0.07	0.03	0.14	0.04	0.12	0.12
Choline O-sulfate	0.17	0.31	0.20	0	0.21	0	0.54	0.55	0.38	0.31	0.37	0.22
Ethanolamine	0.09	0.32	0.29	0.42	0.41	0.73	0.57	0.40	0.40	0.33	0.41	0.35
N-Methylethanolamine	0.16	0.44	0.41	0.46	0.43	0.76	0.74	0.56	0.44	0.37	0.49	0.36
N,N-Dimethylethanolamine	0.45	0.47	0.49	0.73	0.65	0.85	0.87	0.70	0.47	0.40	0.55	0.36

[a]See TABLE 4 for systems.

[b]Abbreviations: t = tailing.

[c]R_f values in first phase.

examined due to its diionic nature. Choline O-sulfate and choline O-phosphate had consistantly low R_f values in both types of systems due to their highly ionic natures while the ethanolamines showed a trend of increasing R_f values as they became increasingly more methylated.

In all cases, the positive identification of unknown choline compounds by paper or thin-layer chromatography required co-chromatography with known standards in several systems since slight variations occurred in the R_f value of the same compound from run to run in the same system due to slight differences in the solvent preparation, chamber saturation, temperature, etc. Such variations in R_f values were found to occur especially in thin-layer systems and thus the use of commercially-prepared plates was deemed a necessity even when unknown samples were co-chromatographed with known standards.

2. Unknown Samples. The Crude Choline Extract from three separate batches of A. carteri was analyzed in a number of paper and thin-layer chromatographic systems. It was found that in 24 paper and 10 thin-layer systems, the Crude Choline Extract always separated into three iodine- and Dragendorff-positive components. That organic components, other than these, were not present in the extract was verified by spraying the silica gel thin-layer chromatograms of the separated components with 50% sulfuric acid. After charring the latter plates, only the same three spots corresponding to the Dragendorff-positive spots were detected.

TABLES 8 and 9 list the R_f values for the three choline components found in the Crude Choline Extract in a number of paper and thin-layer systems and represent an average of at least two chromatographic runs in each system for each batch of cells analyzed. R_f values from run to run for any component seldom deviated by more than 1% from the average. The three sepa-

rated choline compounds are to be referred to as Unknown Components #1, #2 and #3. Unknown Component #3 co-chromatographed in all systems with authentic choline O-sulfate while Unknown Components #1 and #2 occasionally co-chromatographed with known standards but not consistantly enough to warrent identification. However, isolation and rechromatography of these two components (see below) allowed the assignment of the R_f values listed in TABLES 8 and 9 to the two unknowns.

In all paper and thin-layer systems applied to the unknown samples, Unknown Components #1 and #2 always chromatographed in the same sequence, i.e., #1 always had the lower R_f value of the two. Unknown Component #3, however, did not always chromatograph in the same relative position to the other two unknowns.

It was further noted that if one or both of Unknown Components #1 and #2 are choline esters (see below), they must be more polar than esters such as acetyl-, propionyl-, acetyl-β-methyl- and butyrylcholine to account for their relatively low R_f values and thus these two unknown components may contain additional polar function(s) or may contain unsaturation in their acyl moieties.

The reaction of the three components to Dragendorff's Reagent also gave an indication of the character of these compounds. Unknown Component #3 gave a yellow to light orange spot on chromatograms similar to choline O-sulfate while Unknown Components #1 and #2 gave the typical orange color of choline esters. Spraying of the chromatograms with Hanes-Isherwood Reagent revealed the absence of any choline O-phosphate or any other phosphoric acid esters in the Crude Choline Extract from all the batches of A. carteri examined.

TABLE 8.

R_f Values of A. carteri Unknown Choline Components on Paper Chromatography.

System[a]	R_f Value of Unknown Component		
	#1[b]	#2	#3[c]
1	0.27	0.41	0.14
3	0.27	0.40	0.12
6	0.17	0.50	0.35
7	0.18	0.29	0.36
10	0.14	0.29	0.06
13	0.34	0.70	0.59
15	0.09	0.22	0.38
17	0.11	0.23	0.41
19	0.24	0.47	0.12
22	0.35	0.53	0.11
25	0.05	0.52	0.21
26	0.10	0.59	0.32
27	0.05	0.15	0
28	0.04	0.16	0

[a]See TABLE 3.

[b]Co-chromatographs with authentic acrylylcholine.

[c]Co-chromatographs with authentic choline O-sulfate.

TABLE 9.

R_f Values of A. carteri Unknown Choline Components on Thin-Layer Chromatography.

System[a]	R_f Value of Unknown Component		
	#1[b]	#2	#3[c]
4	0.04	0.33	0
6	0.29	0.71	0
7	0.04	0.11	0.53
8	0.12	0.30	0.51
9	0.04	0.14	0.37
10	0.16	0.43	0.31
11	0	0.06	0.35
12	0.13	0.45	0.22

[a]See TABLE 4.

[b]Co-chromatographs with authentic acrylylcholine.

[c]Co-chromatographs with authentic choline O-sulfate.

F. Preparative Paper Chromatography/N. crassa Assay.

In order to determine further whether the three separated components of the Crude Choline Extract were freely-occurring non-lipoidal choline compounds, the extract was analyzed by a combination of preparative paper chromatography and the N. crassa assay. The Crude Choline Extract resulting from the ethanolic extraction of 0.5-1.0 g dry A. carteri cells was run in preparative paper chromatographic systems and the resulting Dragendorff-positive areas of the chromatograms were located by the strip method, sectioned off and eluted according to the methods described above. Solutions from the Dragendorff-positive areas as well as solutions from the remaining Dragendorff-negative areas of the chromatograms were made up to known volumes with distilled water and subjected to the N. crassa assay without prior hydrolysis.

The results of two of these chromatogram/assay experiments are shown in FIGURES 3 and 4. Note that the areas with choline activity on the chromatograms correspond exactly with the areas which are Dragendorff-positive. By referring to TABLE 8, it can be seen that Unknown Component #3, choline O-sulfate, is the slowest spot in FIGURE 3 but the fastest spot in FIGURE 4, while Unknown Components #1 and #2 correspond, respectively, to spots 2 and 3 in FIGURE 3 and spots 1 and 2 in FIGURE 4. The apparent discrepancy with respect to the reversed mycelial responses to Unknown Components #1 and #2 in the two systems may be due to the fact that the two experiments were run on different culture batches of A. carteri.

From the results of these chromatogram/assay experiments, it may be concluded that three non-lipoidal choline compounds occur in A. carteri.

FIGURE 3. Preparative paper chromatography and N. crassa assay of the unhydrolyzed ethanolic extract of A. carteri dry cells in solvent system 1, ethanol:acetic acid:water (90:5:5, v/v) with KCl-treated paper and solvent.

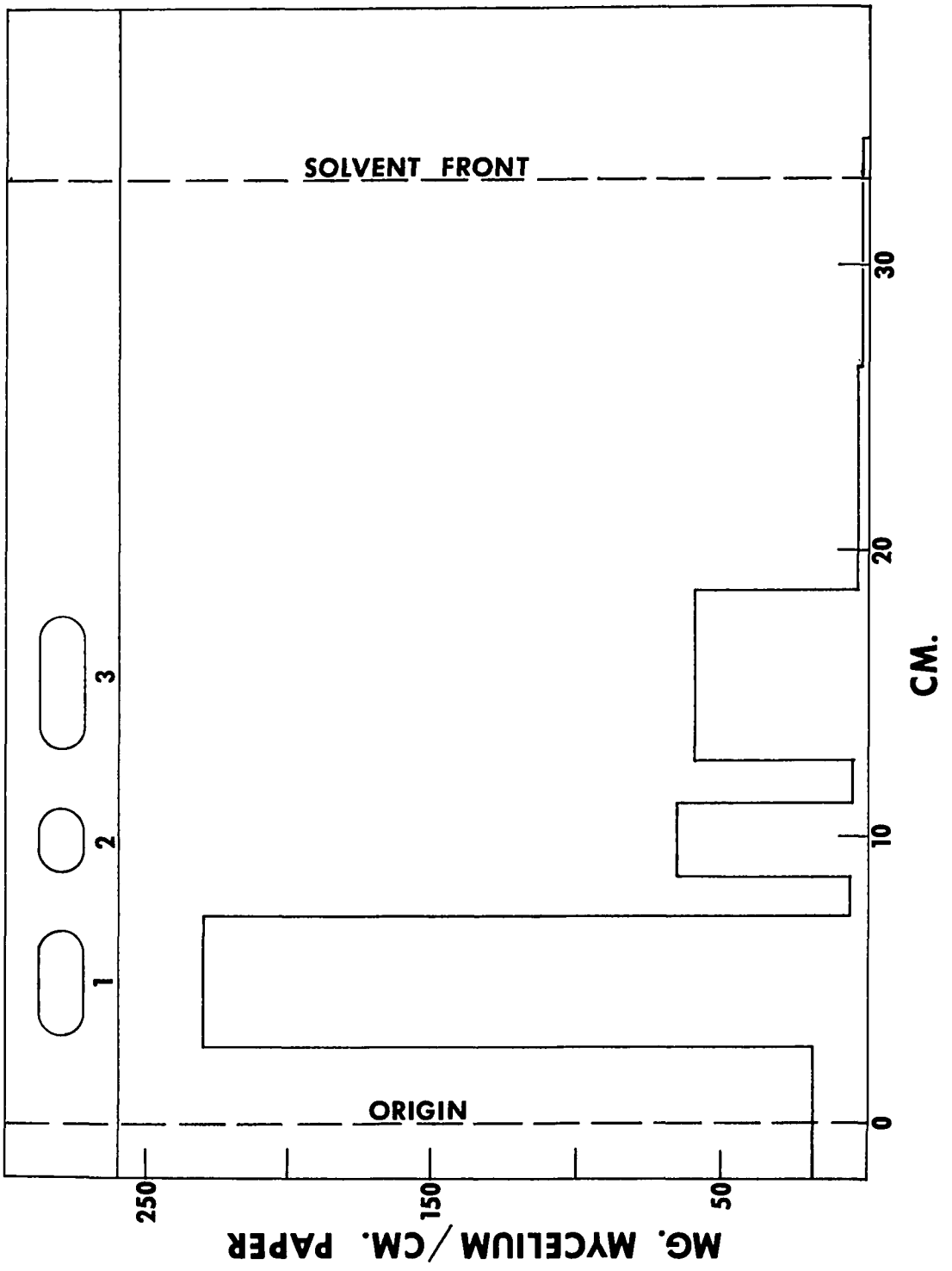
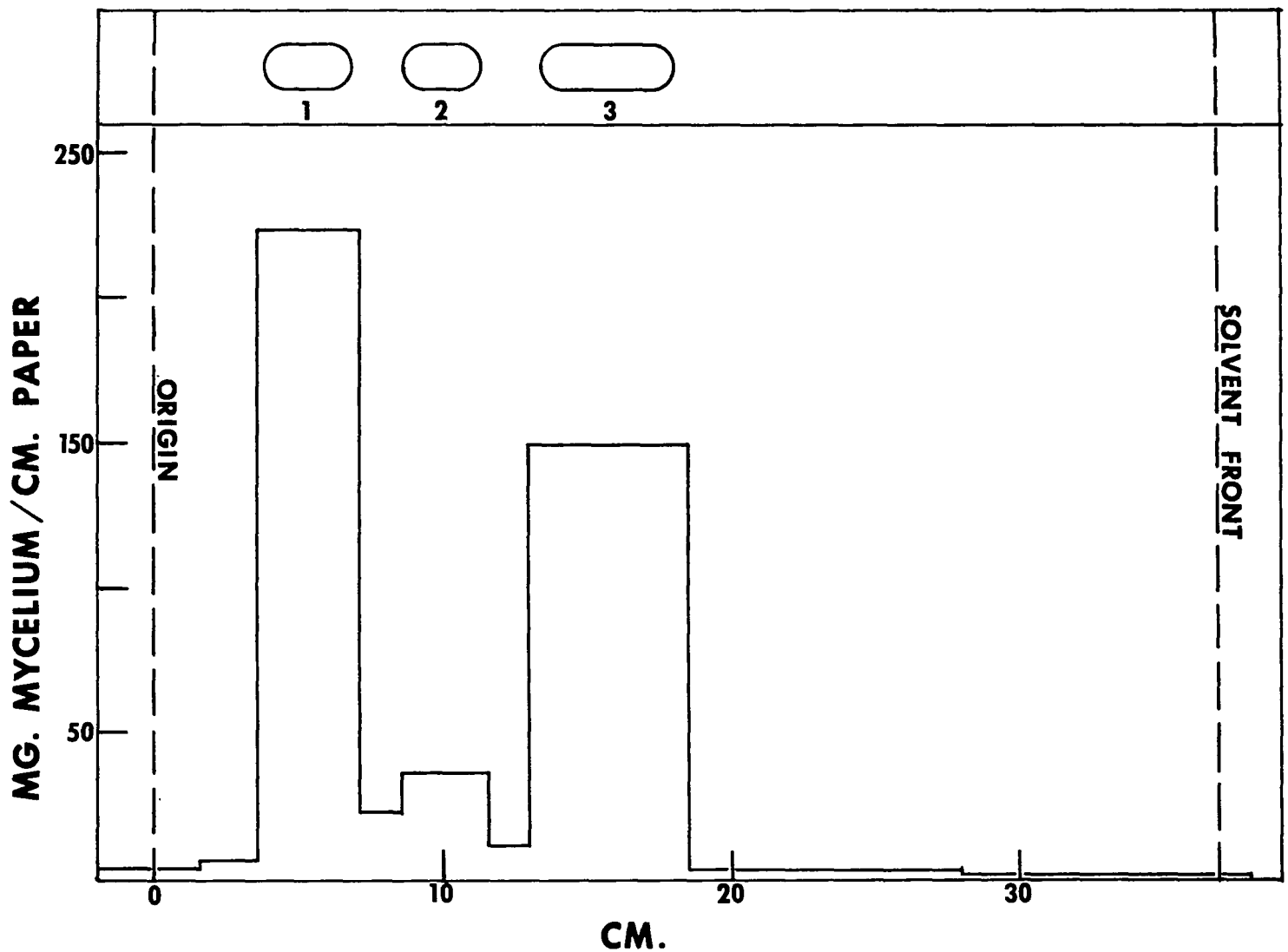

SOLVENT FRONT
ORIGIN
1
2
3
0
10
20
30
CM.
250
150
50
MG. MYCELIUM/CM. PAPER

FIGURE 4. Preparative paper chromatography and N. crassa assay of the unhydrolyzed ethanolic extract of A. carteri dry cells in solvent system 17, 2-propanol:pyridine:water (6:4:3, v/v), no KCl treatment.


1
2
3
250
150
50
ORIGIN
SOLVENT FRONT
MG. MYCELIUM / CM. PAPER
0
10
20
30
CM.

G. Purification of the Unknown Choline Components.

Ion-exchange chromatography was used in initial attempts to separate the three choline components of the Crude Choline Extract. However, it was found that very acidic eluting agents (0.1M acetic acid to 0.5N HCl on the Rexyn 102 carboxylate column; 0.1N HCl on the AG 50W-X4 sulfonic acid column) were required to recover the choline compounds and the resulting low pH's of the eluates made the stability of the suspected choline esters in the Crude Choline Extract questionable. In addition, elution of the columns with appropriate buffers such as 0.1M NaH_2PO_4, which has been used for the recovery of choline esters from ion-exchange columns (53), was also discarded since it proved difficult to separate the resulting eluted choline compounds from the buffer salts. Thus, ion-exchange chromatography was not utilized in the purification of the unknown components.

Preparative paper chromatography in non-KCl systems, however, provided a method for purification of the unknown choline compounds. System 17 (TABLE 3) was used for the separation of the Crude Choline Extract into its three unknown choline components. After the run, the components were located with Dragendorff's Reagent by the vertical strip method described above, eluted from their respective chromatogram areas with water and the extracts were taken to dryness in vacuo. SCHEME 4 illustrates this preparative chromatographic method and lists the typical yields of Unknown Components #1, #2 and #3 from 1.0 g quantities of A. carteri whole dry cells. The typical yields listed in this and following schemes are averages of component quantities found in five extractions of two separate batches of A. carteri. Deviation from the average of any component in any extraction was always less than 10%.

Once the three unknown components were separated, further purification steps were carried out on each of them. Unknown Component #1 was run in a second preparative paper chromatographic system and the Dragendorff-positive

SCHEME 4.

Initial Purification of the Unknown Choline Components from A. carteri.

Crude Choline Extract
(from 1.0 g dry cells)

Preparative Paper Chromatography in System 17

	#1	#2	#3
Weight (mg):	5.6	11.9	26.9
% Weight of whole dry cells:	0.56	1.19	2.69
% Weight after further purification (SCHEMES 5 and 6):	0.23	0.89	0.28

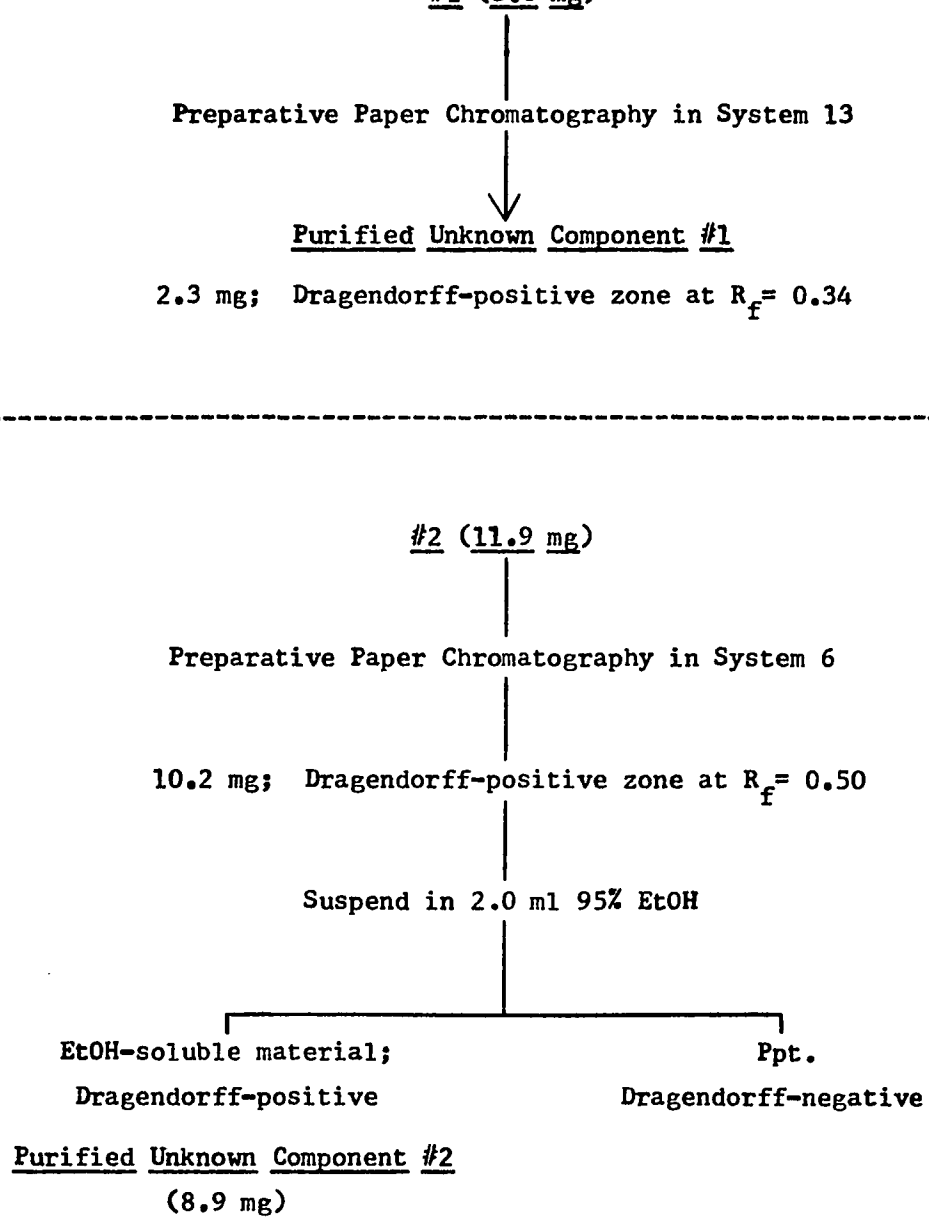
zone was located and eluted, yielding and average of 2.3 mg of chromatographically pure Unknown Component #1 (Purified Component #1, SCHEME 5). This material gave a positive hydroxylamine test which, together with its hydrolysis behavior, Dragendorff color and other evidence (see below), indicated it to be a choline ester.

Unknown Component #2 was also run in a second preparative paper chromatographic system. However, after its elution from the chromatogram and its concentration to dryness in vacuo, it was suspended in 2 ml of 95% ethanol and separated into ethanol soluble and insoluble portions. After centrifuging, only the ethanol-soluble portion of the separation gave a positive Dragendorff reaction upon analysis of both fravtions of the separation in a number of paper and thin-layer systems. The average yield of this Purified Component #2 (see SCHEME 5) was 8.9 mg. The material was extremely hygroscopic and gave a positive hydroxylamine test. This latter result, along with its hydrolysis behavior, Dragendorff color and other evidence (see below), indicated that Unknown Component #2, like Unknown Component #1, is a choline ester.

Separated Unknown Component #3 was suspected of being choline O-sulfate due to its chromatographic behavior so advantage was taken of the latter compound's relative insolubility in ethanol. The dry residue of the chromatographically-isolated unknown was dissolved in 1 ml of distilled water and upon addition of 3 ml of absolute ethanol, a precipitate of pale yellow crystals resulted. A second ethanol precipitation of the ethanol-soluble material using the same method with a smaller volume of water resulted in little additional precipitate. The precipitate was washed and dried and yielded an average of 2.8 mg of Purified Component #3 (see SCHEME 6). The material was Dragendorff-positive and co-chromatographed with known choline O-sulfate in all systems tried with both the standard and unknown samples

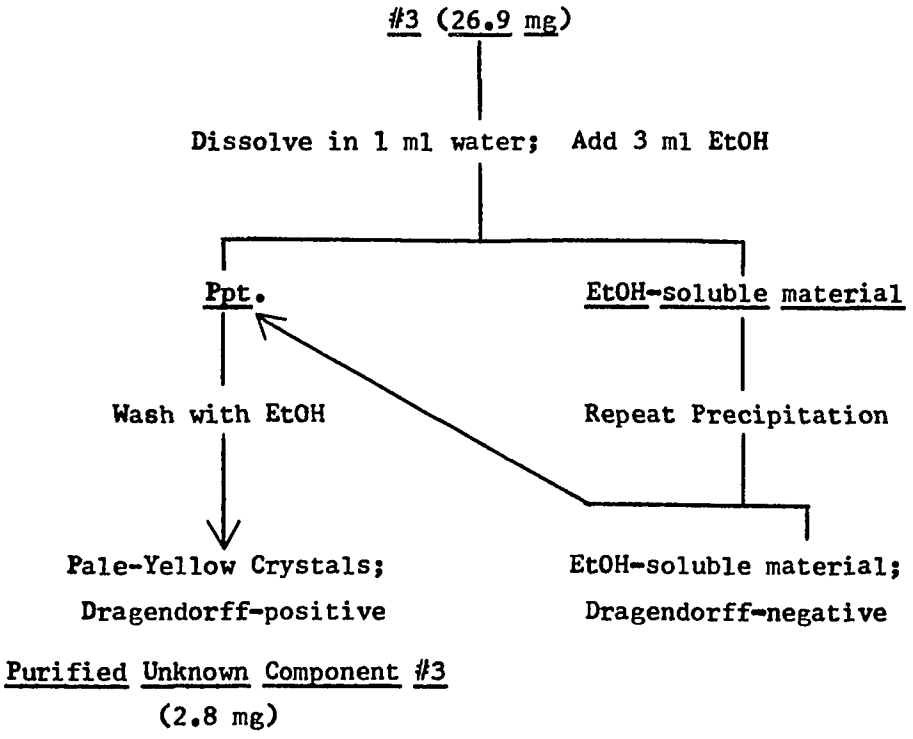
SCHEME 5.

Further Purification of Unknown Choline Components #1 and #2.



SCHEME 6.

Further Purification of Unknown Choline Component #3.



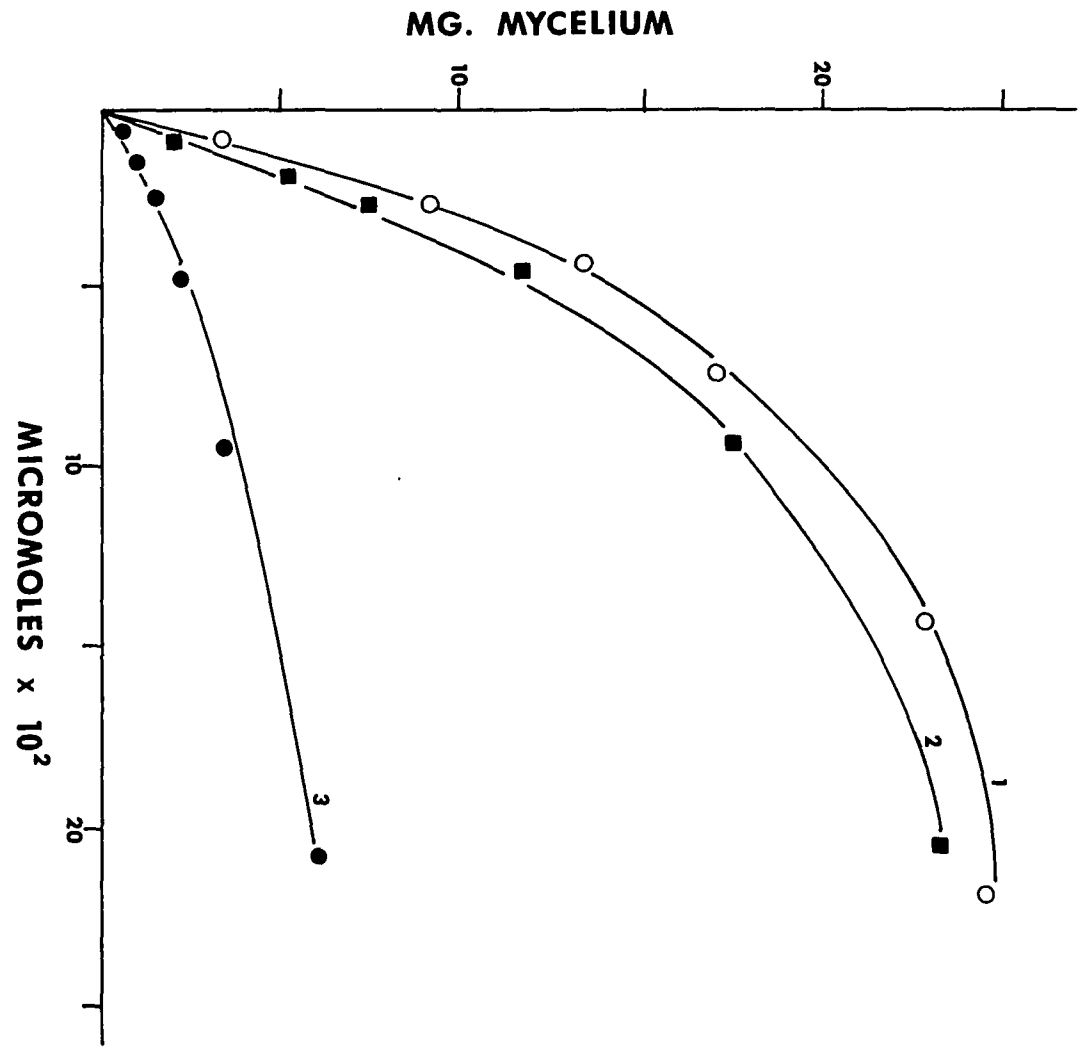
giving dark yellow spots when sprayed with Dragendorff's Reagent. Upon subjecting the unknown crystalline material and known choline O-sulfate to melting point analysis, both the standard and unknown (mixed and in separate capillary tubes) decomposed at 309-310° C in agreement with other reports on the melting characteristics of choline O-sulfate (84). Both the unknown and authentic choline O-sulfate gave a typical high response of choline O-sulfate in the N. crassa assay and both gave negative hydroxylamine tests. These results as well as spectral analysis (see TABLE 15 and FIGURE 9) of Unknown Component #3 are accepted as positive proof that #3 is choline O-sulfate.

H. Acid Hydrolysis and N. crassa Assay of Choline Esters.

It had been observed in N. crassa assays that Unknown Components #1 and #2 gave relatively low responses in the assay compared to Unknown Component #3 on a weight to weight basis. Since #1 and #2 were tentatively identified as choline esters in preceeding work, it was decided to observe whether the activity of these unknowns in the N. crassa assay could be increased by prior hydrolysis and thus add additional proof to the theorized ester nature of the two compounds.

It was found initially by the use of known standards that, whereas esters of choline such as acetyl-, propionyl- and butyrylcholine gave relatively low responses (on a mole to mole basis) in the N. crassa assay in comparison to choline chloride (see FIGURE 1), if these esters were acid hydrolyzed prior to assay, the response they elicited from N. crassa greatly increased due to the liberation of free choline during ester hydrolysis. FIGURE 5 illustrates one such example, that of butyrylcholine before and after acid hydrolysis. As expected, the increase in response in the assay

FIGURE 5. Response of N. crassa to butyrylcholine before and after acid hydrolysis. 1, Choline chloride before and after hydrolysis; 2, butyrylcholine, after hydrolysis; 3, butyrylcholine, before hydrolysis.


MG. MYCELIUM
10
20
MICROMOLES x 10^2
10
20
1
2
3

was less, on a weight to weight basis, as the chain length of the acyl portion of the choline ester increased (see TABLE 10). In all but one case, however (that of acetyl-β-methylcholine), hydrolysis of a choline ester led to a net increase in the N. crassa mycelial response to the ester.

Upon assaying the Crude Choline Extract before and after acid hydrolysis, a 3.5 fold increase in the mycelial response of N. crassa was observed after hydrolysis (see TABLE 10). Since this increase could not be attributed to the action of hydrolysis on choline O-sulfate, it was reasonable to assume that the increased response was due to the hydrolysis of the theorized unknown choline esters #1 and #2. Thus, the three separated unknown components from a preparative paper chromatogram in system 17 were assayed before and after acid hydrolysis. As shown in TABLE 10, Unknown Components #1 and #2 showed approximately 3-fold and 14-fold increases, respectively (see FIGURE 6), in mycelial response from N. crassa after hydrolysis, similar to the responses shown by standard choline esters. Unknown Component #3 gave the expected choline O-sulfate response and showed no increased mycelial response after hydrolysis.

I. Chromatographic Analysis of the Hydrolysis Products of Choline Esters.

In order to further prove that Unknown Components #1 and #2 release free choline upon acid hydrolysis, the residue from hydrolysates of the two unknowns was chromatographed in eight paper chromatographic systems and developed with Dragendorff's Reagent. Propionylcholine chloride and choline chloride (both before and after hydrolysis) were used as standards. The results of one chromatographic system, system 17, are shown in FIGURE 7 while the R_f values of the known and unknown samples before and after

TABLE 10.

Response of Known and Unknown Choline Compounds in the N. crassa Assay Before and After Acid Hydrolysis.

Compound	mg Mycelium/ug Compound Assayed[a]	
	Before Hydrolysis	After Hydrolysis
Choline chloride	2.60	2.70
Acetylcholine	0.71	2.70
Acetyl-β-methylcholine	0.10	0.24
Propionylcholine	0.38	2.42
Acrylylcholine	0.50	1.39
Butyrylcholine	0.28	1.53
Succinyldicholine	0.45	1.65
Choline O-sulfate	1.72	1.73
Choline O-phosphate	0.23	0.29
A. carteri samples		
Crude Choline Extract	0.010	0.035
Butanol/Cell Debris[b]	0	0.005
Unknown Component #1[c]	0.002	0.006
Unknown Component #2[c]	0.005	0.070
Unknown Component #3[c]	0.051	0.053

[a]Total weight assayed: no correction is made for the loss of volatile hydrolysis products.

[b]See SCHEME 2.

[c]Purified to the first preparative chromatogram stage (SCHEME 5).

FIGURE 6. Response of N. crassa to Unknown Component #2 from A. carteri. 1, After acid hydrolysis; 2, before acid hydrolysis.

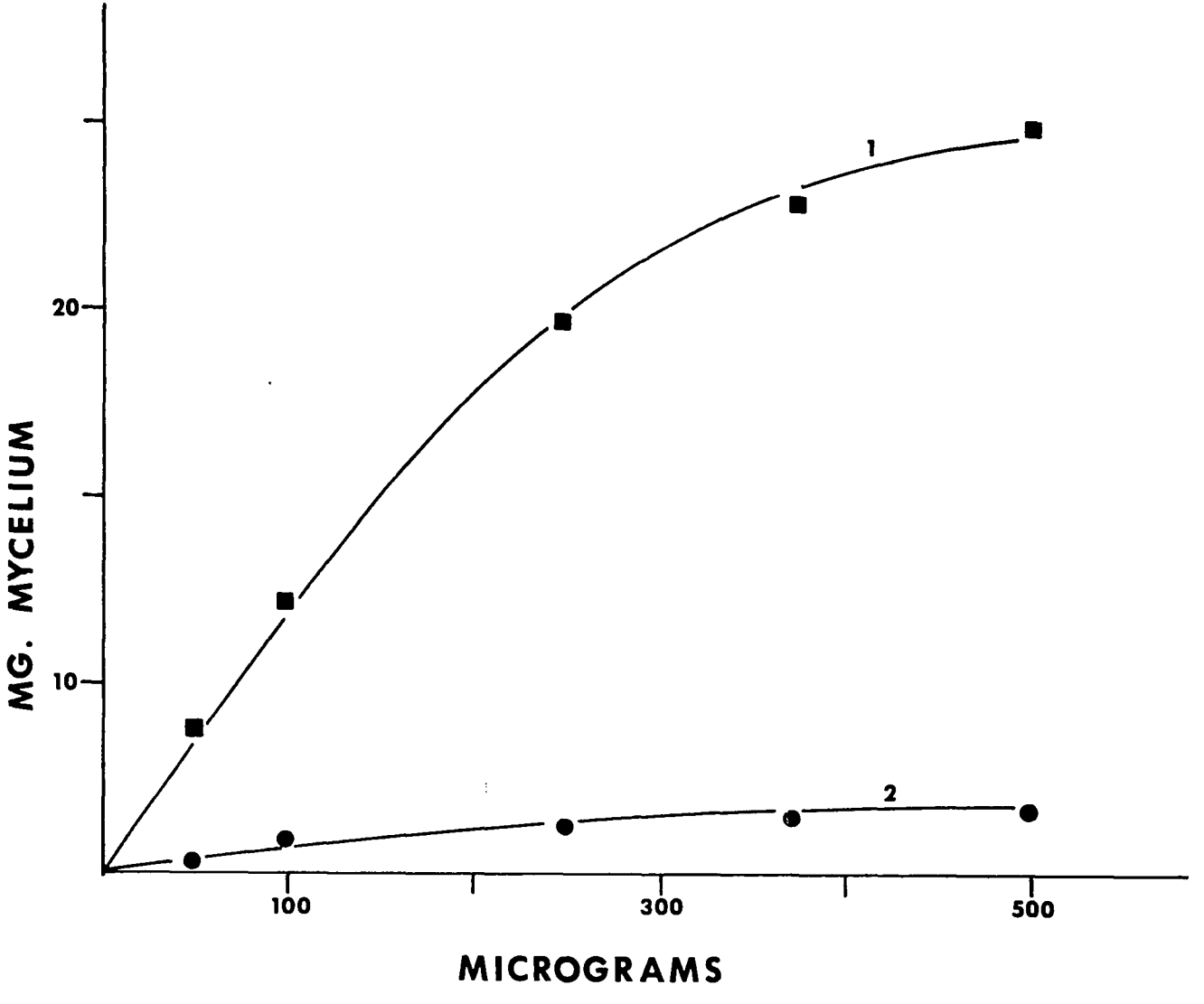
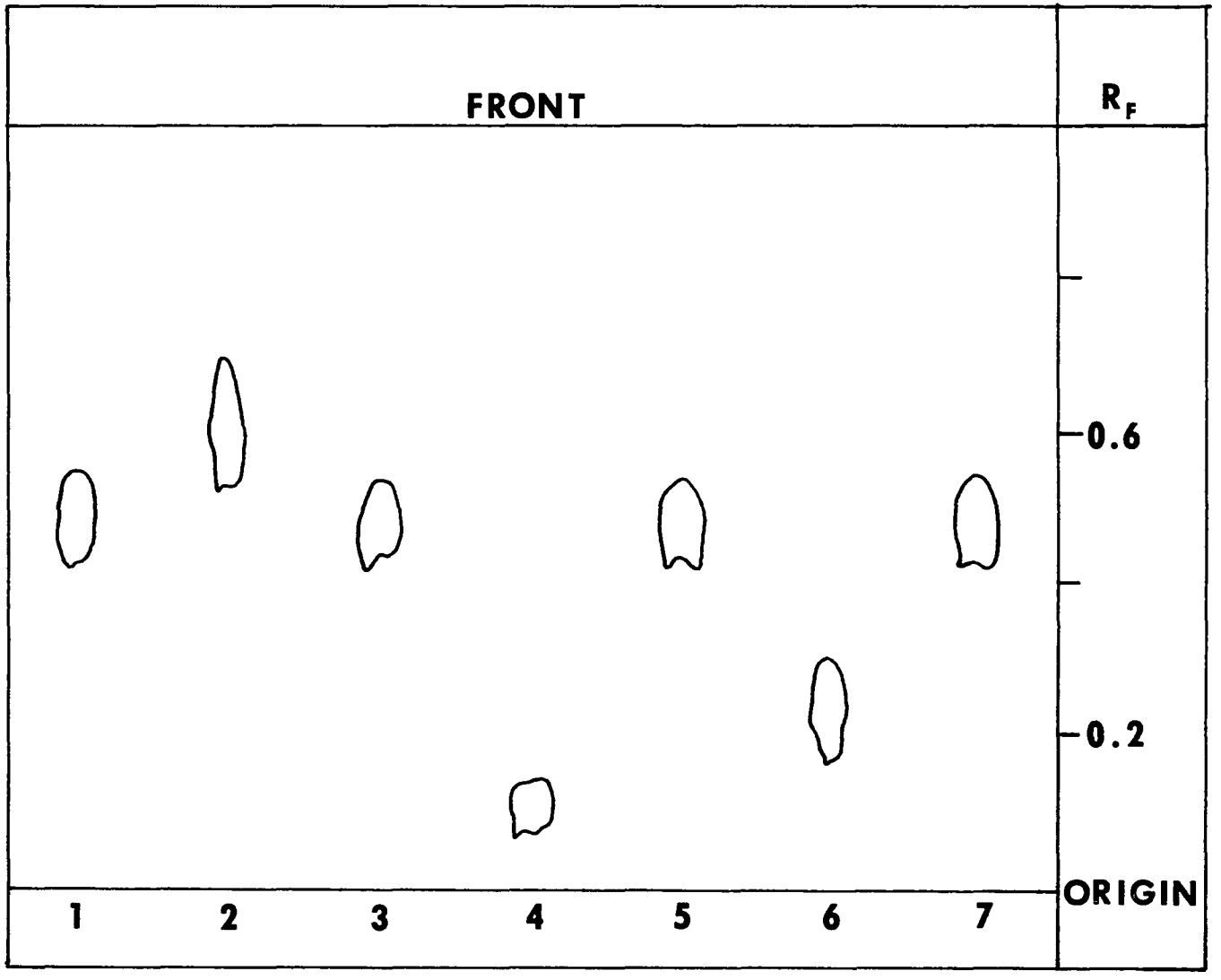

MG. MYCELIUM
20
10
1
2
100
300
500
MICROGRAMS

FIGURE 7. Standard and unknown choline esters before and after acid hydrolysis on paper chromatography in system 17, 2-propanol:pyridine:water (6:4:3, v/v). 1, Choline chloride; 2, propionylcholine chloride; 3, hydrolyzed propionylcholine chloride; 4, Unknown Component #1; 5, hydrolyzed Unknown Component #1; 6, Unknown Component #2; 7, hydrolyzed Unknown Component #2.


FRONT
R_F
0.6
0.2
ORIGIN
1
2
3
4
5
6
7

hydrolysis in the eight systems are reported in TABLE 11. In all systems, the non-volatile hydrolysis product of Unknown Components #1 and #2 co-chromatographed with standard choline chloride and the non-volatile hydrolysis product of propionylcholine.

J. GLC Analysis of Unknown Components #1 and #2.

At present, no successful system is available for the direct identification of choline compounds by gas-liquid chromatography (see 72 and 75 for a review of this area). Thus, a GLC procedure was developed to analyze the free acid components derived from acid hydrolysis of the postulated unknown choline esters #1 and #2. The porous polymer system used, Porapak Q, proved excellent for the separation of short chain compounds with a variety of functional groups. It was decided to standardize the column with a series of short-chain organic acids, alcohols, aldehydes and related compounds. The relative retention times of these compounds are reported in TABLES 12, 13 and 14.

It was found that the Porapak Q column was best suited for compounds containing up to and including five carbon atoms. Compounds with a longer chain length tended to tail excessively and spread at the temperatures used. Most of the standard acids showed good elution patterns and separation from each other (see FIGURE 8). The exceptions were acids with additional functional groups such as pyruvic and lactic acids which spread too much at 200 and 225° C and which tended to tail badly even at the highest temperature used (240° C)(see TABLE 12) for this system to be useful for these compounds. Most of the standard alcohols, aldehydes, etc., could be chromatographed at all temperatures used (see TABLES 13 and 14). As expected, the n-series of alcohols and acids showed a linear relationship

TABLE 11.

R_f Values of Known and Unknown Choline Esters Before and After Acid Hydrolysis on Paper Chromatography.

Substance[a]	R_f Value in System[b]:							
	1	6	10	17	25	26	27	28
Choline (bh; ah)	0.52	0.55	0.40	0.52	0.37	0.44	0.08	0.14
Propionylcholine (bh)	0.68	0.71	0.58	0.63	0.57	0.60	0.22	0.30
Propionylcholine (ah)	0.52	0.55	0.40	0.52	0.37	0.44	0.08	0.14
A. carteri #1 (bh)	0.27	0.15	0.15	0.12	0.05	0.09	0.05	0.03
A. carteri #1 (ah)	0.52	0.55	0.40	0.52	0.37	0.44	0.08	0.14
A. carteri #2 (bh)	0.41	0.47	0.31	0.23	0.51	0.57	0.15	0.16
A. carteri #2 (ah)	0.52	0.55	0.40	0.52	0.37	0.44	0.08	0.14

[a]Abbreviations: bh = before hydrolysis; ah = after hydrolysis.

[b]See TABLE 2.

TABLE 12.

Relative Retention Times of Acids and Unknown Volatile Hydrolysis Products on Porapak Q.

Acid	Relative Retention Time at	
	225°C	240°C
Acetic	1.00[a]	1.00[b]
Propionic	1.74	1.62
Butyric	3.14	2.60
iso-Butyric	2.85	2.47
α-Methylbutyric	5.26 (t)	4.32
Valeric	5.81 (t)	4.51
iso-Valeric	5.46 (t)	4.24
Caproic	nd	8.36 (b)
Acrylic	1.77	1.78
Crotonic	3.90 (t)	3.38
β β-Dimethylacrylic	6.21 (b)	4.81 (t)
Glyoxylic	nd	1.69 (t)
Glycolic	nd	4.33 (b)
Pyruvic	nd	2.23 (t)
Lactic	nd	4.63 (b)
Glyceric	nd	5.25 (b)
Chloracetic	nd	3.89 (b)
Methoxyacetic	nd	3.54 (t)
A. carteri #1[c]	1.77	1.78
A. carteri #2[c]	1.33	1.21

[a]Retention time = 5.71 minutes.

[b]Retention time = 4.53 minutes.

[c]Volatile hydrolysis product.

Abbreviations: t = tailing; b = broad tailing; nd = not determined due to extensive tailing or long retention time.

TABLE 13.

Relative Retention Times of Alcohols and Unknown Volatile Hydrolysis Products on Porapak Q.

Alcohol	Relative Retention Time at		
	200°C	225°C	240°C
Ethyl	1.00[a]	1.00[b]	1.00[c]
Propyl	1.93	1.72	1.59
iso-Propyl	1.52	1.41	1.38
n-Butyl	3.94	3.05	2.61
iso-Butyl	3.38	2.72	2.36
sec-Butyl	3.03	2.46	2.17
t-Butyl	2.22	1.94	1.71
n-Amyl	8.19 (b)	5.69 (t)	4.43
iso-Amyl	7.26 (t)	5.07 (t)	4.07
t-Amyl	4.70	3.58	2.94
A. carteri #1[d]	4.05	3.15	2.87
A. carteri #2[d]	2.66	2.32	2.04

[a]Retention time = 4.18 minutes.

[b]Retention time = 3.12 minutes.

[c]Retention time = 2.82 minutes.

[d]Volatile hydrolysis product.

Abbreviations: t = tailing; b = broad tailing.

TABLE 14.

Relative Retention Times of Miscellaneous Compounds and Unknown Volatile Hydrolysis Products on Porapak Q.

Compound	Relative Retention Time at		
	200°C	225°C	240°C
Acetic Acid	1.00[a]	1.00[b]	1.00[c]
Acrylic Aldehyde	0.62	0.71	0.80
Butyraldehyde	1.23	1.25	1.09
Glycidaldehyde	1.32	1.35	1.36
Crotonaldehyde	1.69	1.64	1.62
Acetone	0.67	0.77	0.84
Methylethyl Ketone	1.22	1.29	1.33
β-Propiolactone	2.52 (t)	2.36	2.27
γ-Butyrolactone	nd	nd	5.50 (t)
A. carteri #1[d]	1.86	1.77	1.78
A. carteri #2[d]	1.16	1.18	1.21

[a]Retention time = 8.82 minutes.

[b]Retention time = 5.35 minutes.

[c]Retention time = 4.35 minutes.

[d]Volatile hydrolysis product.

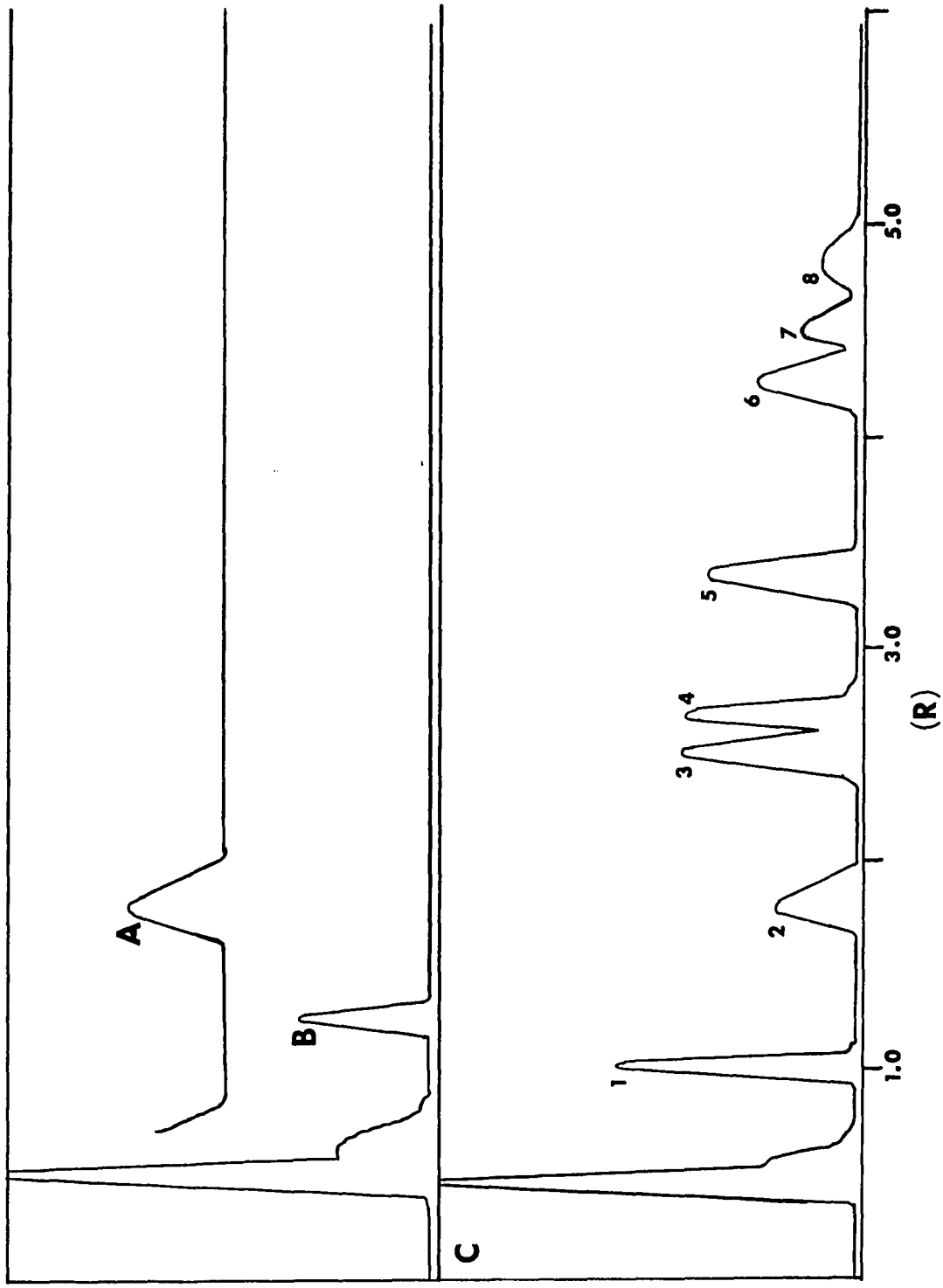
Abbreviations: t = tailing; nd = not determined due to extensive tailing or long retention time.

FIGURE 8. GLC elution patterns for standard acids and derived hydrolysis products from A. carteri Unknown Components #1 and #2 on Porapak Q.

A: Elution peak for the volatile hydrolysis product of Unknown Component #1 at 240°C.

B: Elution peak for the volatile hydrolysis product of Unknown Component #2 at 240°C.

C: Separation of an acid mixture at 240°C. 1, Acetic; 2, acrylic; 3, iso-butyric; 4, butyric; 5, crotonic; 6, iso-valeric; 7, valeric; 8, β,β-dimethylacrylic.


A
B
C
1
2
3
4
5
6
7
8
1.0
3.0
5.0
(R)
DETECTOR RESPONSE, 9 x 10⁻⁹ AMP.

between chain length and column retention time.

Upon acid hydrolysis of Unknown Components #1 and #2 and extraction of the hydrolysates with diethyl ether (see above), GLC analysis of the extracts revealed a single peak for each unknown component, presumably due to the volatile substance liberated from the acyl portion of the hydrolyzed unknown choline ester. The peak resulting from the hydrolysis of Unknown Component #1 showed a retention time and relative retention time identical to that of acrylic acid at all column temperatures (see TABLE 12). Although acrylic and propionic acids have very close retention times at all temperatures, they can be distinguished by the shape of their respective elution peaks, i.e., propionic acid elutes as a sharp, very symmetrical peak while acrylic acid elutes as a slightly asymetrical peak with a rather flattened maximum. The hydrolysis product of Unknown Component #1 also showed this asymmetry and flattening of its elution peak. In addition, co-chromatography of a mixture of the unknown hydrolysis product of #1 with standard acrylic acid showed no peak separation while co-chromatography of the unknown hydrolysis product with propionic acid showed a slight shoulder-like tailing in the otherwise symmetrical elution peak. Based on this GLC analysis of the unknown compound and its hydrolysis products, as well as physiological tests (see below), #1 was thus tentatively identified as acrylylcholine.

When Unknown Component #2 was hydrolyzed and analyzed by GLC, a single, sharp, symmetrical peak resulted, midway between the retention times of acetic and propionic acids. Since the number of volatile 2 and 3 carbon acids is rather limited, the unknown hydrolysis product of #2 was further compared to the alcohols, aldehydes, etc. listed in TABLES 13 and 14. However, no exact correspondence could be found between the unknown hydrolysis

product and any known standard. Thus, these results together with the absence of any known report of a naturally-occurring ether derivative or derivative of choline which would yield an aldehyde or a compound other than an acid upon acid hydrolysis have led to the continued assumption that Unknown Component #2 is a choline ester.

One further observation should be noted here concerning Unknown Component #2. Upon chromatographing a rather old sample of acrylic acid in the GLC system, two peaks resulted. Upon glass redistillation of the acrylic acid to a constant boiling point (141.0°C), the first of these two peaks was found absent upon GLC analysis of the purified acrylic acid. Further analysis with a fresh sample of acrylic acid confirmed that the first peak in the old sample of acrylic acid was a contaminant. However, it was noted with interest that the acrylic acid contaminant present in the old sample of the acid had a retention time which exactly corresponded with the retention time of the hydrolysis product of Unknown Component #2. Thus, it is very possible that Unknown Component #2 contains an acyl group very similar to or derived from acrylic acid. As a check for any possible rearrangements which acrylic acid might undergo during acid hydrolysis, a sample of the pure standard was subjected to the same hydrolysis conditions used on the unknowns. GLC of the resulting acrylic acid extracted from the hydrolysis solution showed no change in retention time.

K. Spectral Analysis of Known and Unknown Choline Compounds.

1. Ultraviolet-Visible Spectra. Due to the absence, in most cases, of conjugation in choline compounds, visible and UV spectra have proven to be of secondary interest in the analysis of these compounds. However, whereas no notable visible spectra (800-350 nm) were exhibited by standard

and unknown choline samples, the UV spectra (350-190 nm) of these compounds proved of interest, especially in the 210-190 nm region. It was found that standard samples of choline and its derivatives give absorbance maxima from 196 to 210 nm and molar absorptivity (ϵ) ranging from 6.8 (choline) to 321 (acetyl-β-methylcholine)(see TABLE 15). Such wavelength maxima and extinctions are not unusual for non-conjugated systems since almost any molecule containing oxygen or nitrogen will absorb near 200 nm due to end absorption ($n \rightarrow n^*$ transitions)(40,112). Thus, choline chloride, choline O-sulfate and choline O-phosphate show absorbance and low extinction values in this region. The esters of choline also have maxima in the 200-210 nm region but their extinction values are greater when compared to, e.g., choline chloride, due to the presence of π electrons in the ester carbonyl groups. Acrylylcholine, containing a conjugated double bond system, showed a double peak spectral curve with $\lambda = 202$, $\epsilon = 13{,}000$ and $\lambda = 225$, $\epsilon = 14{,}000$. However, the second maximum is suspected due to a contaminating polymerization product of acrylylcholine or acrylic acid since absorbance at this wavelength decreased markedly upon purification of the synthesized acrylylcholine (see APPENDIX III). Unknown Component #1, suspected acrylylcholine, exhibited a maximum at 204 nm with a high molar absoptivity (see TABLE 15) as would be expected due to its suspected conjugation. A direct match of the spectra of unknown #1 and authentic acrylylcholine was, however, not possible due to the 225 nm maximum in the spectra of the latter compound. However, a UV analysis was made of the acid liberated upon hydrolysis of Unknown Component #1. It was found that the unknown acid had a $\lambda = 204.5$ nm and $\epsilon = 9428$ in agreement with authentic acrylic acid and with reported values for acrylic acid (112).

UV analysis of Unknown Component #2 resulted in a single peak curve

TABLE 15.

UV Absorption Maxima and Molar Absorptivities for Standard and Unknown Choline Compounds

Compound	λ max[a] (nm)	λ other[a,b] (nm)	ϵ max (moles liter^{-1} cm^{-1})
Choline	200 (s)	221 (b)	6.8
Acetylcholine	206 (b)		60.3
Acetyl-β-methylcholine	204 (s)		321.4
Propionylcholine	207 (b)		77.0
Acrylylcholine	202 (s)	225 (s)	13000
Butyrylcholine	210 (b)		75.0
Succinyldicholine	203 (b)	196 (s)	102.2
Choline O-sulfate	203 (s)	(256)	8.5
Choline O-phosphate	196 (s)	223 (b)	11.7
A. carteri #1	204 (s)		4143[c]
A. carteri #2	202 (s)		246.1[d]
A. carteri #3	203 (s)	(256)	51.8[e]

[a]Abbreviations: s = sharp; b = broad.

[b]Wavelengths in brackets indicate shoulders.

[c]Molecular weight of acrylylcholine used for calculation.

[d]Molecular weight of propionylcholine used for calculation.

[e]Molecular weight of choline O-sulfate used for calculation

with λ = 202 nm and ϵ = 246. The high extinction of this unknown with respect to other cholines may be due either to slight contamination by Unknown Component #1 or due to a chromophore existing in the molecule since it remains a possibility that #2 is derived from acrylylcholine. The possibility of an additional functional group on the molecule such as another oxygen function or a halogen (see 40) may also explain the high extinction of Unknown Component #2. UV analysis of the derived acid from Unknown Component #2 showed an absorption maximum at 205 with ϵ = 50. Such an absorption maximum and extinction is characteristic of free carboxylic acids, e.g., acetic acid has λ = 208 and ϵ =32 (40,112).

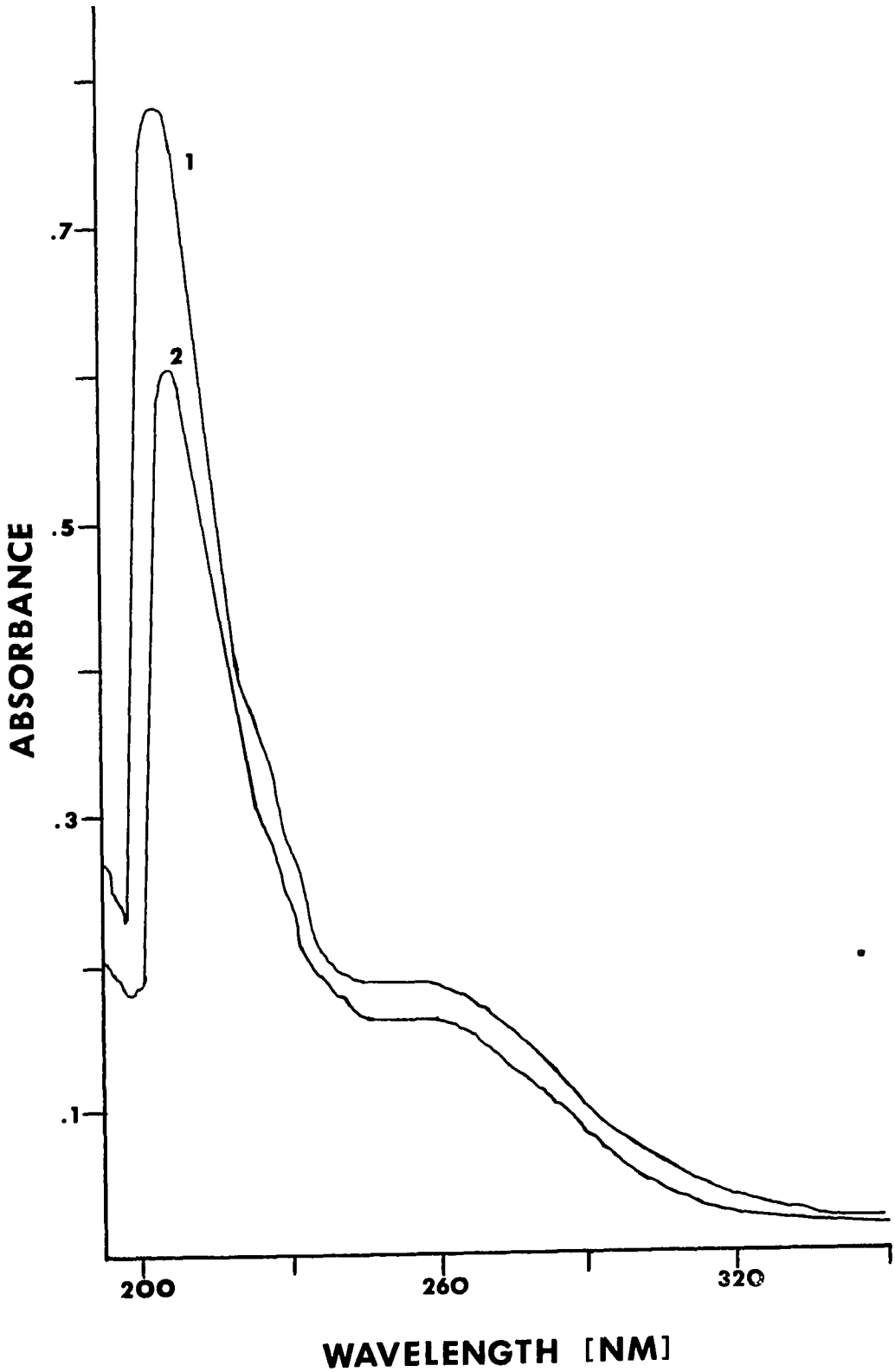
Unknown Component #3 exhibited an absorption curve in the UV identical to that given by standard choline O-sulfate with λ_{max} = 203 and a shoulder at 256 nm (see FIGURE 9). The higher extinction value found with the unknown (TABLE 15) may be assumed due to the cross-contamination of #3 with Unknown Components #1 and #2.

2. Infrared Analysis. Infrared spectral analysis on choline compounds is difficult without previous derivative formation (see 72) due to the extremely deliquescent nature of these compounds. Thus, it was not possible to run infrared spectra on the unknowns due to the rather large amounts of material required for derivative formation. However, IR studies have been made utilizing absolute ethanol as a solvent (48) to study the 2000-1600 cm^{-1} region of choline compounds. Thus, Unknown Component #2 was subjected to such analysis but, unfortunately, the spectra could not be resolved from the bands created by the water present in the sample.

L. Toxicity of Choline Compounds in vivo.

Known choline standards were tested in mice and on fish to determine

FIGURE 9. Ultraviolet absorption spectra of authentic choline O-sulfate (A) and A. carteri Unknown Component #3 (B). Both spectra were taken in 50% ethanol.


ABSORBANCE
.7
.5
.3
.1
1
2
200
260
320
WAVELENGTH [NM]

the minimum lethal dose (MLD) for each compound. The resulting MLD values indicated that these compounds, in mice, are not very potent toxins when compared to such toxins as botulinum or saxitoxin, but they do, nevertheless, illustrate that at relatively low concentrations choline compounds can be toxic *in vivo* in mammals. The results obtained from exposing fish to standard choline compounds are not as clear to interpret and will be discussed below.

The MLD values for standard choline compounds in mice are reported in TABLE 16. Interestingly, acetyl-β -methylcholine appears to be more toxic than acetylcholine in agreement with observations on the effects of choline esters in mammalian systems (79) while succinyldicholine is the most toxic of all the esters tested most likely due to its dicholine nature. Also of interest is the toxicity of acrylylcholine relative to other choline esters. The fact that acrylylcholine was tested as the iodide does not appear relevant since potassium iodide and sodium iodide are only toxic at much higher doses than those found for acrylylcholine (117a). Choline O-sulfate and choline O-phosphate showed no toxicity at levels of 1000 mg/kg and are thus assumed non-toxic with respect to other choline compounds.

When known choline compounds were dissolved in artificial sea water and killifish were placed in the resulting solutions, it was found that the fish were not seriously affected by concentrations of 10 mg/ml and 5 mg/ml of acetyl- and acetyl-β -methylcholine, respectively. Succinyldicholine, however, killed the fish within 10 minutes at concentrations greater than 7.5 mg/ml and caused the fish to lie helpless in the water for 1-2 hours at concentrations greater than 5.0 mg/ml. These results are interesting since our original observations of the toxicity of *A. carteri* late culture

TABLE 16.

Toxicity of Choline Compounds in Mice

Compound	MLD/20 g[a]	MLD/kg[a]
Choline chloride	5.0	250
Acetylcholine chloride	2.5	125
Acetyl-β-methylcholine bromide	1.75	87.5
Propionylcholine chloride	6.5	325
Acrylylcholine iodide	1.25	62.5
Butyrylcholine chloride	7.5	375
Succinyldicholine dichloride	0.05	2.5
Choline O-sulfate	> 20.0	> 1000
Choline O-phosphate	> 20.0	> 1000

[a]Minimum Lethal Dose (mg) needed to kill a 20 ± 2 g mouse within 24 hours when injected interperitoneally in a volume of 0.25 ml water.

supernates showed that such supernates were toxic to killifish. Possible explanations for these appearingly conflicting results include (a) the late culture supernates of A. carteri might contain choline compounds at concentrations greater than 10 mg/ml or might contain compounds as active or more active than succinyldicholine; (b) the saturated esters tested may not be effective as neurotoxins on marine animals; or (c) in order for the A. carteri choline compounds to act as toxins, they must be ingested by the fish. Of these three postulates, the second can be considered further since choline esters of marine origin appear to be much different than those of mammalian origin, i.e., the former esters are mainly derived from acrylic acid and may have branched or heterocyclic acyl groups while the latter all have saturated straight chain acyl ester groups. Of interest here are the results of studies on unsaturated choline esters, which have shown that distinct physiological differences exist between choline esters which possess unsaturated acyl groups and their saturated counterparts (107,108). Unfortunately, due to the limited amounts of unsaturated choline esters which could be produced by standard synthesis methods, these compounds could not be tested with fish to either support or disprove the second postulate.

A limited amount of in vivo testing was done with various fractions from the ethanolic extraction procedure used on A. carteri dry cells (SCHEME 2). It was found that all fractions of the extraction were non-toxic in mice at a level of 1250 mg/kg except the Crude Choline Extract. This latter fraction caused death in mice in 4-12 hours at a level of 1000 mg/kg while 750 mg/kg and 500 mg/kg levels caused initial convulsions and then relaxation in the animals with recovery occurring in 1-2 hours. There was not enough of the extraction fraction material to test with killifish.

Thus, it appears that the toxicity of A. carteri cells is located in

the Crude Choline Extract and, presumably, in the choline compounds of the extract. As yet, insufficient amounts of the individual Unknown Choline Components have prevented testing then in vivo.

M. Physiological Tests of the Unknown Choline Components.

The following physiological tests were applied to the Crude Choline Extract and Purified Component #1 (SCHEME 5), acrylylcholine. Detailed results and interpretations of these tests have been reported elsewhere (104,125), and thus the following results are summaries of the test results.

1. Mercenaria mercenaria Assay. The myogenic heart of the bivalve Mercenaria (Venus) mercenaria has been shown to be extremely sensitive to acetylcholine (56,100), responding to amounts of the ester as little as 10^{-12} g/ml. The acetylcholine appears to act on the pacemaker of the clam heart and is believed to be the normal cardiac inhibitory neurohumor of this organism. Other studies have supported this theory and have shown that the heart is very specific in that it responds only to choline esters, responding to carbamyl-, propionyl-, butyryl- and acetyl-β-methylcholine (although higher concentrations of these esters are required to elicit a comparable acetylcholine response), but not appreciably to choline itself or to esters such as benzoylcholine (133).

Utilizing isolated and in vivo Mercenaria hearts, it was shown that acetylcholine at concentrations ranging from 10^{-9} to 10^{-7} g/ml caused a decrease in the frequency and amplitude of the hearts. If the hearts were pretreated with 10^{-5} g/ml of Mytolon (benzoquinonium chloride), an anticholinergic which can block the action of acetylcholine on cholinergic receptors at concentrations as low as 10^{-8} g/ml (49,131), then the acetylcho-

line had no effect on the heart.

Similar results on Mercenaria hearts were shown by samples of A. carteri choline compounds. Crude Choline Extract at a concentration of 10^{-2}g/ml and Purified Component #1 at a concentration of 2.2 x 10^{-5}g/ml both caused a decrease in the frequency and amplitude of the isolated clam heart in five minutes with diastolic arrest occurring as a result of either sample in 20 minutes. In vivo hearts also showed this decrease in frequency and amplitude upon treatment with 5 x 10^{-3}g/ml of Crude Choline Extract with the effect being revesible upon washing. If the isolated or in vivo hearts were pretreated with 10^{-5}g/ml of Mytolon, neither the Crude Choline Extract nor Purified Component #1 showed any effects on the preparations.

2. Decapod Crustacean Assays. The neurogenic heart of the decapods Carcinus maenas and Cancer irroratus are stimulated by acetylcholine at concentrations as low as 10^{-9}g/ml (130). When the in vivo hearts of these animals were treated dropwise with 10^{-3}g/ml of the A. carteri Crude Choline Extract or 2.2 x 10^{-4}g/ml of Purified Component #1, a two-fold increase in the heart frequency resulted which was reversible with sea water washing. If the hearts were treated with 10^{-2}g/ml of the Crude Choline Extract, irrevesible systolic arrest resulted.

3. Rana pipiens Assay. The heart of the frog, Rana pipiens, is myogenic and, similar to all vertebrates, is inhibited by acetylcholine released from the inhibitory vagus nerve. When 5 mg of Crude Choline Extract was injected into a frog heart, a typical acetylcholine-like response resulted with a decrease in heart frequency. Injection of 10 mg of crude extract resulted in diastolic arrest. When 0.2 ml of a 10^{-3}g/ml solution of atropine sulfate (which blocks the action of acetylcholine on vertebrate

hearts) was injected into a heart prior to injection of the Crude Choline Extract, the latter substance had no effect on the heart frequency.

4. Mouse Intestine Assay. The use of guinea pig and mouse intestine as an assay system for acetylcholine and related choline esters is well known (see, e.g., 31,77), the application of these substances resulting in contraction of the smooth muscle. It was found, using an isolated strip of mouse intestine, that 0.5×10^{-3} g/ml of A. carteri Crude Choline Extract and 1.25×10^{-4} g/ml of Purified Component #1 stimulated a two-fold increase in the frequency of intestine contractions in five minutes as well as a 50% increase in the amplitude of the contractions. These increases in frequency and amplitude were comparable to those induced by a standard 10^{-6} g/ml sample of acetylcholine. The effects of all the samples on the intestine were reversible with washing.

It thus appears that the components in the Crude Choline Extract of A. carteri and most certainly Unknown Component #1 (acrylylcholine) are acting as typical choline esters in physiological test systems.

N. Comparison of Unknown Component #1 to Authentic Acrylylcholine.

To attempt positive identification of Unknown Component #1 as acrylylcholine, the latter compound was synthesized as described below (APPENDIX III) and compared to Unknown Component #1. The synthesized acrylylcholine was initially judged pure by physical and spectral methods although it was necessary to purify the compound further in order to ascertain that it was chromatographically-pure (see APPENDIX III). When the purified acrylylcholine was assayed with N. crassa, it gave the typical low response of a choline ester before acid hydrolysis and an increased response almost equal

to that of standard choline chloride after acid hydrolysis. Furthermore, its hydrolysis products were found to be choline chloride (via paper chromatography) and acrylic acid (via GLC analysis). UV analysis results of the prepared compound have been discussed above. The prepared acrylylcholine also gave a positive hydroxylamine test. Thus, it was concluded that the synthesized compound was, indeed, acrylylcholine.

The standard acrylylcholine was co-chromatographed with Purified Unknown Component #1 (SCHEME 5) in the 14 paper and 8 thin-layer chromatographic systems listed in TABLES 8 and 9. In all these systems, the standard and unknown had identical R_f values when sprayed with Dragendorff's Reagent and gave the typical red-orange color of choline esters with the reagent. Thus, it is concluded that Unknown Component #1 is acrylylcholine.

IV. CONCLUSION.

The present study has resulted in the discovery and characterization of three non-lipoidal choline compounds in the marine dinoflagellate, Amphidinium carteri. Two of the compounds have been identified as choline O-sulfate and acrylylcholine while the other is, as yet, an unidentified choline ester which appears related to acrylylcholine. A summary of the properties of these three choline compounds is presented in TABLE 17.

The suspected choline O-sulfate from A. carteri has been shown to exhibit the same chromatographic, spectral, chemical and physical properties of authentic choline O-sulfate. In addition, the A. carteri compound elicits the same nutritional response in the N. crassa assay as authentic choline O-sulfate.

The suspected acrylylcholine from A. carteri behaves in an identical manner with respect to chromatographic, chemical and physical analysis methods as authentic acrylylcholine. In addition, the A. carteri acrylylcholine behaves as a typical choline ester in both the N. crassa assay as well as in physiological test systems specifically sensitive to choline esters.

The unidentified choline ester from A. carteri (Unknown Component #2) appears to be derived from acrylylcholine based on the gas chromatographic behavior of its hydrolysis product. Thus, it is postulated to contain either two or three carbon atoms in its acyl group and to most likely contain an additional heteroatom functional group to account for its chromatographic and spectral characteristics. The possibility may also still exist that Unknown Component #2 is not an ester but rather an ether or other derivative of choline. However, the absence of any report of such choline derivatives

TABLE 17.

Summary of the Properties of the A. carteri Unknown Choline Components.

Analysis	Unknown Component		
	#1	#2	#3
Hydroxylamine test	+	+	-
Melting Point	Hygroscopic	Extremely hygroscopic	309-310°C (decomposes)
Response to N. crassa assay[a]			
1. Before hydrolysis	0.04/ug	0.05/ug	0.65/ug
2. After hydrolysis	0.12/ug	0.70/ug	0.65/ug
Chromatographic behavior	Dragendorff (+), ester indicated; Low R_f; releases choline upon hydrolysis; co-chromatographs with authentic acrylylcholine	Dragendorff (+), ester indicated; Low R_f; releases choline upon hydrolysis	Dragendorff (+) with yellow color; co-chromatographs with authentic choline O-sulfate

[a] ug choline chloride euivalence/ug purified unknown.

TABLE 17 (Continued).

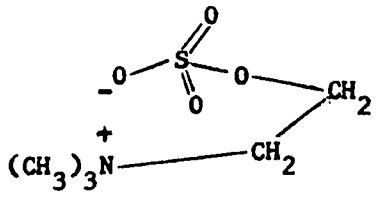
Analysis	Unknown Component		
	#1	#2	#3
GLC Analysis	Volatile hydrolysis product co-chromatographs with standard acrylic acid	Volatile hydrolysis product has t midway between acetic and propionic acids	No volatile hydrolysis product
UV Spectra	λ_{max} = 204 nm	λ_{max} = 202 nm	λ = 203; (256) nm
	ϵ_{max} = 4143	ϵ_{max} = 246	ϵ_{max} = 51.8
Identification	Acrylylcholine	Choline ester with 2-3 carbon acyl group	Choline O-sulfate

from natural sources would tend to negate such a non-ester theory. In addition, the hydroxylamine, chromatographic and hydrolysis results reported above appear to support the ester theory for Unknown Component #2.

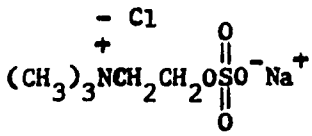
The occurrence of non-lipoidal choline compounds in a marine dinoflagellate has not, up to the present time, been reported, even though one might expect such compounds to exist in these primary marine food chain sources since higher marine organisms contain many quaternary ammonium compounds. The obvious question thus arises concerning the function of these choline substances in a marine alga. Choline O-sulfate can be postulated as serving three possible functions in living organisms; (a) that of a transport factor of sulfate, (b) as a source of sulfate, and/or (c) as an osmoregulator. The first two functions appear directly linked to each other in all investigations to date. Choline O-sulfate has been shown to be an actively transported molecule in bacterial and algal cells and may also serve as the sole source of sulfate in these organisms (19, 114). Supporting this observation in lower organisms, it has been observed that choline O-sulfate is also actively taken up and transported in a number of plant systems (95,96) and may act as an effective transport agent for sulfate and possibly other ions in choline-containing membranes. It might be noted here that although these first two postulated functions for choline O-sulfate appear linked in all systems examined thus far, the possibility of finding an active transport function for the compound without a sulfatase function in an organism is still plausible if, for example, choline O-sulfate serves a function other than purely as a source of sulfate. Thus, these two functions can be considered mutually exclusive. In any case, since A. carteri has a rather high nutritional requirement for sulfate (see APPENDIX I), a possible role of choline O-sulfate in the trans-

port of and as a source of sulfate in the alga could thus be postulated.

The third possible function of choline O-sulfate, that of an osmoregulator, could explain the transport of salt ions by choline O-sulfate out of cells producing the substance. Choline O-sulfate may be postulated as occurring in two possible forms *in vivo*, (a) as a cyclic structure (117,142) by virtue of an intramolecular salt bridge between the quaternary nitrogen and sulfate group:



or (b) as the salt of an appropriate ion pair, such as sodium chloride:



In the latter case, it could be assumed that such an ion-bearing form of choline O-sulfate would act in the removal of the salt ions from inside the cell by actively transporting them through the cell membrane and out of the cell. Such a theory of active salt transport out of cells be choline O-sulfate is supported by recent observations that certain mangrove plant species which actually excrete 2M NaCl at their leaf surfaces produce large amounts of choline O-sulfate (which is found excreted with the salt) while species of the same plants which do not excrete salt do not produce choline O-sulfate (20). Thus, applying such a function for choline O-sulfate to *A. carteri*, it would follow that such a compound could play an

important role in actively transporting salts out of the alga. Certainly such a theory is supported by the postulated role of other quaternary bases found in marine organisms, such as homarine, as osmoregulators (see above).

As was discussed above, many, if not all, of the choline esters as well as many of the non-choline quaternary ammonium compounds found in marine organisms, may act as potential toxins. Thus, it is possible that the two choline esters found in A. carteri serve as defensive substances against parasites and predators. Such a theory of choline esters functioning as toxic factors is supported by the action of quaternary ammonium compounds on, for example, mollusks (128); by our own results on the effects of choline esters, such as succinyldicholine, on fish; and by reports concerning the activity of alcoholic extracts of a strain of A. carteri (Amphidinium carteri Hulburt) as antibacterial agents against species of Staphylococcus and marine species of Corynebacterium, Brevibacterium, Micrococcus and Flavobacterium (39). Of course, the mechanism of toxicity of choline esters would be expected to vary between the microorganisms and marine animals mentioned, i.e., whereas choline compounds might be acting as toxins on bacteria and other microorganisms by some unknown mechanism, they most likely act as neurotoxins in animal systems.

While the above examples illustrate the possible primary roles of choline esters in the marine environment, these compounds may also exert a secondary influence once they enter into the food chain. Thus, the occurrence of acrylylcholine in higher marine organisms, e.g., in species of Buccinidae (137), might be due to the concentration of the choline ester from ingestion of algae such as A. carteri which produce the compound or by ingestion of another animal which feeds on such choline ester-producing algae. In addition, choline esters produced by algae such as A. carteri

may be hydrolyzed by esterases to choline after ingestion by, e.g., members of the Pelecypoda, and lead to the large and toxic accumulations of choline found, for example, in the ovaries of the bivalve Callistra brevisiphonata (10) and in short-necked clams (123). Certainly one example which can be cited of the movement of a toxic compound up the marine food chain is that of acrylic acid itself, which is known to occur in some algae (8,109,110,111) and which has antimicrobial activity. Concentration of acrylic acid produced by the alga Phaeocystis occurs in the gastrointestinal tract of penguins which feed on crustacea which feed on the alga and leads to the suppression of coliform microflora in the penguins (110). Other examples of the movement of toxic substances up the marine food chain were cited above with respect to saxitoxin and other substances. Thus, it is very possible that the choline esters produced by A. carteri are produced as defensive substances primarily directed against bacteria, zooplankton and possibly some fish, but are also able to move up the marine food chain per se and act secondarily as neurotoxins in higher marine organisms.

The discovery of choline compounds in a dinoflagellate opens a wide range of possible studies on these organisms to determine whether toxicity in many of them can be linked to the production of quaternary ammonium compounds. Thus, utilizing the methods described, further studies may be warranted with other marine algae which are suspected as being toxic in the marine environment.

BIBLIOGRAPHY.

1. Ackermann, D. 1927. Über die Identität das Aktinins mit dem γ-Butyrobetain. Z. Biol. 86: 199-202.

2. Ackermann, D. 1953. Über das Vorkommen von Homarin, Trigonellin und einer neun base Anemonin in der Anthrozoe Anemonia sulcata. Z. Physiol. Chem. 295: 1-9.

3. Ackermann, D. and P.H. List. 1958. Über das Vorkommen von Herzynin, Ergothionein, Homarin, Trigonellin, Glykollbetain, Cholin, Trimethylamin, Adenin und fast sämtlicher Amionosäuren des Eiweisses in Limulus polyphemus. Z. Physiol. Chem. 313: 30-36.

4. Ackermann, D. and P.H. List. 1959. Über das Vorkommen von Taurobetain, Taurin und Inosit im Risenkielschwamm. Z. Physiol. Chem. 317: 78-81.

5. Ackermann, D. and R. Janka. 1953. Konstitution und Synthese des Anemonins. Z. Physiol. Chem. 294: 93-97.

6. Ackermann, D., F. Holtz and H. Reinwein. 1923. Reindarstellung und Konstitutionsermittelling des Tetramins, eines giftes aus Aktinia equina. Z. Biol. 79: 113-120.

7. Ackermann, D., F. Holtz and H. Reinwein. 1924. Über die Extraktstoffe von Aktinia equina. Z. Biol. 80: 131-136.

8. Allen, M.B. and E.Y. Dawson. 1960. Production of antibacterial substances by benthictropical marine algae. J. Bacteriol. 79: 459-460.

9. Ariens, E.J. and A.M. Simonis. 1967. Cholinergic and anti-cholinergic drugs, do they act on common receptors? Ann. N.Y. Acad. Sci. 144: 842-868.

10. Asano, M. 1954. Occurrence of choline in the shellfish Callistra brevisiphonata Carpenter. Tohoku J. Agr. Res. 4: 239-250.

11. Asano, M. and M. Itoh. 1960. Salivary poison of a marine gastropod, Neptunea arithritica, and the seasonal variation of its toxicity. Ann. N.Y. Acad. Sci. 90: 674-688.

12. Augustinsson, K. and M. Grahn. 1953. The separation of choline esters by paper chromatography. Acta Chem. Scand. 7: 906-912.

13. Babo, L. and M. Hirschbumn. 1852. Über das Sinapin. Ann. Chem. Liebig's 84: 10-32.

14. Banister, J., V.P. Whittaker and S. Wijesundera. 1953. The occurrence of homologs of acetylcholine in ox spleen. J. Physiol. (London) 121: 55-71.

15. Baslow, M.H. 1969. Marine Pharmacology. The Williams & Wilkins Co., Baltimore.

16. Baeyer, A. 1866. Synthese des Neurins. Ann. Chem. Liebig's 140: 306-313.

17. Baeyer, A. 1867. Über das Neurin. Ann. Chem. Liebig's 142: 322-326.

18. Beers, J.R. 1967. The species distribution of some naturally occurring quaternary ammonium compounds. Comp. Biochem. Physiol. 21: 11-21.

19. Bellenger, N., P. Nissen, T.C. Wood and I.H.Segel. 1968. Specificity and control of choline O-sulfate transport in filamentous fungi. J. Bacteriol. 96: 1574-1585.

20. Benson, A.A. and M.R. Atkenson. 1967. Choline sulfate and phosphate in salt excreting plants. Fed. Proc. 26: #772.

21. Bischoff, C., W. Grab and J. Kapfhammer. 1931. Acetylcholin im Rinderblut. Z. Physiol. Chem. 199: 135-168.

22. Bischoff, C., W. Grab and J. Kapfhammer. 1932. Acetylcholin im Warmbluter. Z. Physiol. Chem. 207: 57-77.

23. Bisset, G.W., J.F.D. Frazer, M. Rottschild and M. Schachter. 1960. A pharmacologically active choline ester and other substances in the garden tiger moth, Arctia caja (L.). Proc. Roy. Soc. (London) B152: 255-262.

24. Bowman, W.C., B.A. Hemsworth and M.J. Rand. 1967. Effects of analogs of choline on neuromuscular transmission. Ann. N.Y. Acad. Sci. 144: 471-482.

25. Boylan, D.B. and P.J. Scheuer. 1967. Pahutoxin: A fish poison. Science 155: 52-56.

26. Bremer, J. and D.M. Greenberg. 1959. Mono- and dimethylethanolamine isolated from rat-liver phospholipids. Biochim. Biophys. Acta 35: 287-288.

27. Bremer, J., P.H. Figard and D.M. Greenberg. 1960. The biosynthesis of choline and its relation to phospholipid metabolism. Biochim. Biophys. Acta 43: 477-488.

28. Brimblecombe, R.W. and D.G. Rowswell. 1969. A comparison of the pharmacological activities of tertiary bases and their quaternary ammonium derivatives. Int. J. Neurochem. 8: 131-141.

29. Brundish, D.E. and J. Baddiley. 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. 110: 573-582.

30. Bull, G., C. Hebb and D. Morris. 1969. Synthesis of acetylcholine in the electric organ of Torpedo. Comp. Biochem. Physiol. 28: 11-28.

31. Chang, H.C. and J.H. Gaddum. 1933. Choline esters in tissue extracts. J. Physiol. (London) 79: 255-285.

32. Ciereszko, L.S., P.H. Odense and R.W. Schmidt. 1960. Chemistry of coelenterates II. Occurrence of taurobetaine and creatine in gorhonians. Ann. N.Y. Acad. Sci. 90: 920-922.

33. Craig, L.E. 1948. Cuariform activity and chemical structure. Chem. Revs. 42: 285-410.

34. Dale, H.H. 1914. The action of certain esters of choline and their relation to muscarine. J. Pharmacol. Exptl. Therap. 6: 147-190.

35. Dale, H.H. and H.W. Dudley. 1929. The presence of histamine and acetylcholine in the spleen of the ox and horse. J. Physiol. (London) 68: 97-123.

36. Dale, H.H. and W. Feldberg. 1934. The chemical transmitter of vagus effects to the stomach. J. Physiol. (London) 81: 320-334.

37. de Flines, J. 1955. The occurrence of a sulfuric acid ester of choline in the mycelium of a strain of P. chrysogenum. J. Amer. Chem. Soc. 77: 1676-1677.

38. Dubois, R. 1903. Sur le venin de la glande a pourpee des murex. Compt. Rend. 55: 81.

39. Duff, D.C.B., D.L. Bruce and N.J. Antia. 1966. The antibacterial activity of marine planktonic algae. Can. J. Microbiol. 12: 877-884.

40. Dyer, J.R. 1965. Applications of Absorption Spectroscopy of Organic Compounds. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

41. Engel, R.W., W.D. Salmon and C.J. Ackerman. 1954. Chemical estimation of choline. Methods in Biochemical Analysis 1: 265-286.

42. Erspamer, V. and O. Benati. 1953. Isolierung des Murexins aus Hypobranchialdrusenextrakten von Murex trunculus und seine Identifizierung als β-(Imidazolyl-4(5))-acryl-cholin. Biochem. Z. 324: 66-73.

43. Erspamer, V. and O. Benati. 1953. Identification of murexine as β -(imidazoyl-(4))-acrylylcholine. Science 117: 161-162.

44. Ewins, A.J. 1914. Acetylcholine, a new active principle of ergot. Biochem. J. 8: 44-49.

45. Fänge, R. 1957. An acetylcholine-like salivary poison in the marine gastropod, Neptunea antiqua. Nature 180: 196-197.

46. Fänge, R. 1960. The salivary gland of Neptunea antiqua. Ann. N.Y. Acad. Sci. 90: 689-694.

47. Feldberg, W. and J.H. Gaddum. 1934. The chemical transmitter at synapses in a sympathetic ganglion. J. Physiol. (London) 81: 305-319.

48. Fellman, J.H. and T.S. Fujita. 1962. The conformation of acetylcholine. Biochim. Biophys. Acta 56: 227-231.

49. Florey, E. 1967. The clam heart bioassay for acetylcholine. Comp. Biochem. Physiol. 20: 365-377.

50. Florey, E. 1967. Neurotransmitters and modulators in the animal kingdom. Fed. Proc. 26: 1164-1178.

51. Frankel, G. 1953. Studies on the distribution of vitamin B_T (carnitine). Biol. Bull. 104: 359-371.

52. Frankel, G. 1954. The distribution of vitamin B_T (carnitine) throughout the animal kingdom. Arch. Biochem. Biophys. 50: 486-495.

53. Gardiner, J.E. and V.P. Whittaker. 1954. The identification of propionylcholine as a constituent of ox spleen. Biochem. J. 58: 24-29.

54. Gasteiger, E.L., P.C. Haake and J.A. Gergen. 1960. An investigation of the distribution and function of homarine (N-methyl picolinic acid). Ann. N.Y. Acad. Sci. 90: 622-636.

55. Goldfine, H. 1962. The characterization and biosynthesis of N-methylethanolamine phospholipid from Clostridium butyricum. Biochim. Biophys. Acta 59: 504-506.

56. Greenberg, M.J. 1965. A compendium of responses of bivalve hearts to acetylcholine. Comp. Biochem. Physiol. 14: 513-539.

57. Griffith, W.H. and J.F. Nyc. 1971. Choline VII. Estimation. In: The Vitamins (W.H. Sebrell, Jr. and R.S. Harris, eds.), Vol. III, pp. 70-75. Academic Press, New York.

58. Halstead, B.W. 1965. Poisonous and Venomous Marine Animals of the World Vol. I. Invertebrates. U.S. Gov't. Printing Office, Washington, D.C.

59. Hanes, C.S. and F.A. Isherwood. 1949. Separation of phosphoric esters on filter paper chromatogram. Nature 164: 1107-1112.

60. Harada, T. and B. Spencer. 1960. Choline sulfate in fungi. J. Gen. Microbiol. 22: 520-527.

61. Hartman, W.J., W.G. Clark, S.D. Cyr, A.L. Jordon and R.A. Leibhold. 1960. Pharmacologically-active amines and their biogenesis in the octopus. Ann. N.Y. Acad. Sci. 90: 637-666.

62. Haurowitz, F. and H. Waelsch. 1926. Über die chemische Zusammensetzung der Qualle Velella spirans. Z. Physiol. Chem. 161: 300-317.

63. Hebb, C.O. 1957. Biochemical evidence for the neural function of acetylcholine. Physiol. Rev. 37: 196-220.

64. Hestrin, S. 1949. The reaction of acetylcholine and other varboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. 180: 249-261.

65. Hoppe-Seyler, F.A. 1933. Über das Homarin, eine bisher unbekannte tierische Base. Z. Physiol. Chem. 222: 105-115.

66. Horowitz, N.H. and G.W. Beadle. 1943. A microbiological method for the determination of choline by use of a mutant of Neurospora. J. Biol. Chem. 150: 325-333.

67. Hosein, E.A. and A. Orzeck. 1964. Some physiological and biochemical properties of acetyl-L-carnitine isolated from brain tissue extracts. Int. J. Neuropharmacol. 3: 71-76.

68. Hosein, E.A. and P. Proulx. 1960. Isolation and probable functions of betaine esters in brain metabolism. Nature 187: 321-322.

69. Hosein, E.A., P. Proulx and R. Ara. 1962. Substances with acetylcholine activity in normal rat brain. Biochem. J. 83: 341-346.

70. Hunt, R. and Taveau, R. de M. 1911. The effects of a number of derivatives of choline and analogous compounds on the blood pressure. Bull. U.S. Publ. Helth. Serv. (Hyg. Lab.), No. 73. U.S. Gov't Printing Office, Washington, D.C.

71. Ikawa, M. 1967. Bacterial phosphatides and natural relationships. Bacteriol. Rev. 31: 54-64.

72. Ikawa, M. and R.F. Taylor. Choline and related substances in algae. In: Marine Pharmacognosy (D.F. Martin and G.M. Padilla, eds.), Academic Press, New York, in press.

73. Ikawa, M., P.J. Borowski and A. Chakravarti. 1968. Choline and inositol distribution in algae and fungi. App. Microbiol. 16: 620-623.

74. Ikawa, M., A. Chakravarti and R.F. Taylor. The occurrence of choline in Lactobacillus plantarum. Can. J. Microbiol., in press.

75. Jenden, D.J. and L.B. Campbell. 1971. Measurement of choline esters. Methods in Biochemical Analysis (supplement): 183-216.

76. Kewitz, H. 1959. Nachweis von 4-Amino-n-butyrylcholin im Warmblutergehirn. Arch. Exp. Pathol. Pharmacol. 237: 308-318 (Biol. Abstr. 40: #18549 (1962)).

77. Keyl, M.S., I.A. Michaelson and V.P. Whittaker. 1957. Physiologically active choline esters in certain marine gastropods and other marine invertebrates. J. Physiol. (Lonfon) 139: 434-454.

78. Kohn, A.J., P.R. Saunders and S. Wiener. 1960. Preliminary studies on the venom of the marine snail Conus. Ann. N.Y. Acad. Sci. 90: 706-725.

79. Kranjevic, K. and J.W. Phillips. 1963. Pharmacological properties of acetylcholine-sensitive cells in the cerebral cortex. J. Physiol. (London) 166: 328-350.

80. Kuehl, Jr., F.A., N. Lebel and J.W. Richter. 1955. Isolation and characterization studies on muscarine. J. Amer. Chem. Soc. 77: 6663-6665.

81. Kuriaki, K., T. Yakushiji, T. Noro, T. Shimizu and S. Saji. 1958. Gamma-aminobutyrylcholine. Nature 181: 1336-1337.

82. Liebreich, O. 1865. Über die chemische Beschaffenheit der Gehirnsubstanz. Ann. Chem. Liebig's 134: 29-44.

83. Lindberg, B. 1955. Methylated taurines and choline sulphate in red algae. Acta Chem. Scand. 9: 1323-1326.

84. Lindberg, B. 1955. Studies on the chemistry of lichens. VIII. Investigation of a Dermatocarpion and some Rocella species. Acta Chem. Scand. 9: 917-919.

85. Loewi, O. 1921. Über humorale Übertragbarkeit der Herznervenwirkung. Pfluger's Arch. Ges. Physiol. 189: 239-242.

86. Maizel, J.V., A.A. Benson and N.A. Tolbert. 1956. Identification of phosphoryl choline as an important constituent of plant saps. Plant Physiol. 31: 407-408.

87. Marchbanks, R.M. and M. Israel. 1971. Aspects of acetylcholine metabolism in the electric organ of Torpedo manorata. J. Neurochem. 18: 439-448.

88. Mathias, A.P., D.M. Ross and M. Schachter. 1958. Distribution of histamine, 5-hydroxytryptamine, tetramethylammonium and other substances in coelenterates possessing nematocysts. J. Physiol. (London) 142: 56-57P.

89. Mathias, A.P., D.M. Ross and M. Schachter. 1960. The distribution of 5-hydroxytryptamine, tetramethylammonium, homarine and other substances in sea anemones. J. Physiol. (London) 151: 296-311.

90. McFarren, E.F., H. Tanabe, F.J. Silva, W.B. Wilson, J.E. Campbell, and K.H. Lewis. 1965. The occurrence of a ciguatera-like poison in oysters, clams and Gynodinium breve cultures. Toxicon 3: 111-123.

91. McLaughlin, J.A. and L. Provasoli. 1957. Nutritional requirements and toxicity of two marine Amphidinium. J. Protozoo. 4: 7S.

92. Melville, D.C. 1959. Ergothionine. Vitamins and Hormones 17: 155-204.

93. Mendes, E.G., L. Abbud and S. Umiji. 1963. Cholinergic action of homogenates of sea urchin pedicellariae. Science 139: 408-409.

94. Nakazawa, Y. 1959. Studies on the new glycolipide in oyster. V. The nitrogenous components and structure of the glycolipide. J. Biochem. (Tokyo) 46: 1579-1585.

95. Nissen, P. and A.A. Benson. 1961. Choline sulfate in higher plants. Science 134: 1759.

96. Nissen, P. and A.A. Benson. 1964. Active transport of choline sulfate by barley roots. Plant Physiol. 39: 586-589.

97. Ogata, A. and T. Komoda. 1943. Components of Echinarachnius mirabilis and Echnoidea echinoderma. J. Pharm. Soc. Japan 63: 653-658 (Chem. Abstr. 45: #2943 (1951)).

98. Pentraeth, V.W. and G.A. Cottrell. 1968. Acetylcholine and cholinesterase in the radial nerve of Asterias rubens. Comp. Biochem. Physiol. 27: 775-785.

99. Pfeiffer, C.C. 1948. Nature and spatial relationship of the prosthetic chemical groups required for maximal muscarinic action. Science 107: 94-96.

100. Prosser, C.L. 1940. Acetylcholine and nervous inhibition in the heart of Venus mercenaria. Biol. Bull. 78: 92-102.

101. Reissman, W. and TH. Wieske. 1967. Detection of N-methylated ethanolamines in phosphatide hydrolysates by thin-layer chromatography. Analyt. Biochem. 19: 46-49.

102. Remsen, I. and R.D. Coale. 1884. Investigations on sinapine. I. On sinapic acid. Amer. Chem. J. 6: 52-60.

103. Roaf, H.E. and M. Nierenstein. 1907. The physiological action of the extract of the hypobranchial gland of Purpura lapillus. J. Physiol. (London) 36: 5-8.

104. Sasner, Jr., J. Comparative studies on algal toxins. In: Marine Pharmacognosy (D.F. Martin and G.M. Padilla, eds.), Academic Press, New York, in press.

105. Schantz, E.J., J.D. Mold, D.W. Stanger, J. Shavel, F.J. Riel, J.P. Bowden, J.M. Lynch, R.S. Wyler, B. Riegel and H. Sommer. 1957. Paralytic shellfish poison VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissues. J. Amer. Chem. Soc. 79: 5230-5235.

106. Schmidt, R.W. and L.S. Ciereszko. 1968. Chemistry of coelenterates IX. Occurrence of choline sulfate and scyllitol in the gorgonian Erythropodium caribaeorum. Proc. Okla. Acad. Sci. 48: 258-259.

107. Sekul, A.A. and W.C. Holland. 1961. Comparative pressor effect of certain unsaturated acid esters of choline. J. Pharmacol. Exptl. Therp. 133: 313-318.

108. Sekul, A.A., W.C. Holland and R. House. 1963. Relationship between electronic structure and intensity of nicotine-like action of choline esters. Arch. Int. Pharmacodyn. 141: 404-411.

109. Sieburth, J. McN. 1960. Acrylic acid, an "antibiotic" principle in Phaeocystis blooms in antarctic waters. Science 132: 676-677.

110. Sieburth, J. McN. 1961. Antibiotic properties of acrylic acid, a factor in the gastrointestinal antibiosis in polar marine animals. J. Bacteriol. 82: 72-79.

111. Sieburth, J. McN. 1964. Antibacterial substances produced by marine algae. Develop. in Indust. Microbiol. 5: 124-135.

112. Silverstein, R.M. and G.C. Bassler. 1968. Spectrometric Identification of Organic Compounds. John Wiley & Sons, Inc., New York.

113. Smith, I. (ed.) 1969. Chromatographic and Electrophoretic Techniques. Vol. I. Chromatography. 3rd Edition, John Wiley & Sons, Inc., New York.

114. Spencer, B. and T. Harada. 1960. The role of choline sulphate in the sulfur metabolism of fungi. Biochem. J. 77: 305-315.

115. Stahl, E. (ed.). 1969. Thin-Layer Chromatography, A Laboratory Handbook. Springer-Verlag, Inc., New York.

116. Stephenson, M. and E. Rowatt. 1947. The production of acetylcholine by a strain of Lactobacillus plantarum. J. Gen. Microbiol. 1: 279-298.

117. Stevens, C.M. and P. Vohira. 1955. Occurrence of choline sulfate in P. chrysogenum. J. Amer. Chem. Soc. 77: 4935-4936.

117a. Strecher, P.G. 1968. The Merck Index. Merck & Co., Inc., Rahway, New Jersey.

118. Strecker, A. 1849. Beobachtungen über die Galle verschiedner Thiere. Ann. Chem. Liebig's 70: 149-197.

119. Strecker, A. 1862. Über einige neue Bestandtheile der Schweingalle. Ann. Chem. Liebig's 123: 353-360.

120. Sullivan, G. and L.R. Brady. 1965. Thin-layer chromatographic separation of betaine, choline and muscarine. Lloydia 28: 68-70.

121. Takemoto, T., K. Daigo and N. Takagi. 1964. Studies on the hypotensive constituents of marine algae I. A new basic amino acid "laminine" and other basic constituents from Laminaria angustata. Yakugaku Zasshi 84: 1176-1179.

122. Taylor, E.H. 1964. Thin-layer chromatography of choline and derivatives. Lloydia 27: 96-99.

123. Terada, Y. and I. Hitoshi. 1970. Chemical and pharmacological studies on putrefactive short-neck clam. Nippon Yakurigaku Zasshi 66: 304-315.

124. Thomson, D.A. 1964. Ostracitoxin: An ichthyotoxic stress secretion of the boxfish, Ostracion lentiginosus. Science 146: 244-245.

125. Thurberg, F.P. 1972. Comparative effects of aquatic biotoxins on cardiac systems. Ph.D. Dissertation, University of New Hampshire.

126. Tomasz, A. 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. Science 157: 694-697.

127. Uvnäs, B. 1960. Mechanism of action of histamine liberating principle in jellyfish (Cyanea capillata). Ann. N.Y. Acad. Sci. 90: 751-759.

128. Vallejo-Freire, A., O.F. Ribeiro and I.F. Ribeiro. 1953. Quaternary ammonium compounds as molluscacides. Science 119: 470-472.

129. Wangersky, P.J. and R.R.L. Guillard. 1960. Low molecular weight organic base from the dinoflagellate Amphidinium carteri. Nature 185: 689-690.

130. Welsh, J.W. 1939. Chemical mediation in crustaceans I. The occurrence of acetylcholine in nervous tissues and its actions on the decapod heart. J. Exptl. Biol. 16: 198-219.

131. Welsh, J.W. 1954. Marine invertebrate preparations useful in the bioassay of acetylcholine and 5-hydroxytryptamine. Nature 173: 955-956.

132. Welsh, J.W. and P.G. Prock. 1959. Quaternary ammonium bases in coelenterates. Biol. Bull. 115: 551-561.

133. Welsh, J.W. and R. Taub. 1948. The action of choline and related compounds on the heart of Venus mercenaria. Biol. Bull. 95: 346-353.

134. Welsh, J.W. and R. Taub. 1950. Molecular configuration and biological activity of substances resembling acetylcholine. Science 112: 467-469.

135. Whittaker, V.P. 1951. Specificity, mode of action and distribution of cholinesterase. Physiol. Rev. 31: 312-343.

136. Whittaker, V.P. 1957. β,β-Dimethylacrylylcholine, a new naturally-occurring physiologically active ester of choline. Biochem. J. 66: 35P.

137. Whittaker, V.P. 1960. Pharmacologically active choline esters in marine gastropods. Ann. N.Y. Acad. Sci. 90: 695-705.

138. Whittaker, V.P. and I.A. Michaelson. 1954. Studies on urocanylcholine. Biol. Bull. 107: 304.

139. Whittaker, V.P. and S. Wijesundera. 1952. The separation of esters of choline by paper chromatography. Biochem. J. 51: 348-351.

140. Whittaker, V.P. and S. Wijesundera. 1952. The hydrolysis of succinyldicholine by cholinesterase. Biochem. J. 52: 475-479.

141. Winkler, L.R., B.E. Tilton and M.G. Hardinge. 1962. A cholinergic agent extracted from sea hares. Arch. Int. Pharmacodym. 137: 76-83.

142. Wolley, D.W. and W.H. Peterson. 1937. The chemistry of mold tissue XIV. Isolation of a cyclic choline sulfate from A. sydowi. J. Biol. Chem. 122: 213-218.

143. Wurtz, M.A. 1867. Synthése de la névrine. Compt. Rend 65: 1015-1016.

144. Wurtz, M.A. 1868. Sur l'identité de la névrine artificelle avec la névrine naturelle. Compt. Rend. 66: 772-776.

APPENDIX I.

Growth and Harvesting of Amphidinium carteri.

A. carteri was grown in unialgal culture in sterile 20 liter glass carboys at 24 ± 2°C. The media utilized was NH-15 as described by Gates and Wilson, 1960 (private communication), as follows:

1. Initial Components

Compound	g/l
NaCl	24.0
KCl	0.6
$MgCl_2 \cdot 6H_2O$	4.5
$MgSO_4 \cdot 7H_2O$	6.0
$CaCl_2$	0.7

2. Add 977 ml distilled water and:

   1 ml of a 1% solution of K_2HPO_4

   1 ml of a 1% solution of KNO_3

   1 ml of a 1% solution of EDTA

   5 ml of a 10% solution of tris buffer

   5 ml of Metals Solution (see below)

   5 ml of Sulfides Solution (see below).

3. Stir and then autoclave at 15 psi for 15 minutes. Cool and pass the media through a 0.45 u Millipore filter. Add to the filtered media:

   1.0 ml of a 1% solution of thiamine

   0.1 ml of biotin solution (0.5 mg biotin/100 ml water)

   0.1 ml of Vitamin-8-Mix (see below)

0.2 ml of a 0.5% solution of adenine sulfate

0.1 ml of B_{12} solution (1 mg Vitamin B_{12}/100 ml water)

4. Adjust the pH of the media to 8.0 with concentrated HCl and allow the media to sit overnight at 24°C before inoculation.

Metals Solution

Compound	g/l
Fe Tartrate	0.250
H_3BO_3	0.300
H_2SeO_3	0.010
NH_4VO_3	0.012
K_2CrO_4	0.011
$MnCl_2$	0.037
$TiCl_2$	0.083
Na_2SiO_3	0.500
$ZrOCl_2$	0.040
$BaCl_2$	0.015

Sulfides Solution

Compound	g/l
NH_4Cl	1.00
KH_2PO_4	0.50
$MgCl_2$	0.20
$NaHCO_3$	1.00
$Na_2S \cdot 9H_2O$	0.75

Vitamin-B-Mix

Compound	mg/l
Thiamine	200.00
Biotin	0.50
Vitamin B_{12}	0.05
Folic Acid	2.50
PABA	10.00
Thymine	800.00
Choline	500.00
Inositol	1000.00
Putrescine	8.00
Riboflavin	5.00
Pyridoxine	40.00
Pyridoxamine	20.00
Orotic Acid	260.00
Nicotinic Acid	100.00

The media was inoculated from existing cultures of A. carteri to result in an initial concentration of 6-12 million cells per liter. The carboys were placed next to vertically-mounted double 40 watt white flourescent lights such that the center of each carboy was approximately 12 inches from the light (ca., 250 foot candles). The cultures were continuously illuminated and aerated during growth.

During growth, the cultures were monitored daily with a Model F Coulter Counter until they reached a peak density as described by a previously determined standard growth curve of the organism. At this time,

the cells were harvested by continuous flow centrifugation utilizing a Sorvall RC2-B refrigerated centrifuge equipped with a Sorvall KSB continuous flow system at 15°C and 5000 rpm (3020 x g) and with a continuous flow rate of 0.366 liters/minute. The resulting cell pellet was washed once with distilled water, lyophilized and stored in the deep freezer. Yields were approximately 1 gm dry cells per 8-10 liters of culture.

APPENDIX II.

Modified Malt Extract Agar[a].

Component	g/l
Malt Extract (Difco)	10.0
Peptone (Difco)	0.5
Glucose (Fisher)	10.0
Agar (Difco)	10.0

The components were dissolved in 1000 ml of distilled water, the resulting solution was distributed in screw-capped tubes, and the tubes were autoclaved at 15 psi for 15 minutes.

---

[a]The modification is of Blakeslee's original formula which calls for twice the amounts of the components per liter then reported above (Raper, K.B. and D.I. Fennell. 1965. The genus Aspergillus. Williams and Wilkins Co., Baltimore, p. 38).

APPENDIX III.

Synthesis of Acrylylcholine.

Acrylylcholine was synthesized in the laboratory of Dr. Kenneth Andersen, Department of Chemistry, University of New Hampshire, according to a method adapted from other workers[a]. Addition of 25.2 g (0.35M) acrylic acid to 41.5 g (0.35M) thionyl chloride with subsequent heating resulted in acrylylchloride. This compound was added to a diethyl ether solution of 26.8 gm (0.30M) N,N-dimethylethanolamine. After neutralizing the solution with dilute sodium hydroxide, the product was extracted with diethyl ether, dried over $MgSO_4$ and the ether evaporated off *in vacuo*. The resulting oil was distilled at 38-44°C and 0.8mm and 3 ml of acrylic acid N,N-dimethylaminoethyl ester was collected. The ester was then dissolved in diethyl ether and treated with methyl iodide. Acrylylcholine iodide precipitated from the solution immediately. After a series of recrystallizations, 0.0068 g of product was collected (m.p. = 133.5-135°C).

The synthesis product was observed to have two distinct components in a number of paper and thin-layer chromatographic systems, a slow moving component corresponding to *A. carteri* Unknown Component #1 and a fast moving component with a high R_f value with respect to standard choline esters. Both components were iodine and Dragendorff-positive with the slower moving component giving an orange (typical of choline esters) and the faster component a blue-red (also characteristic of some choline esters) Dragendorff color. The two components were separated from each other by

---

[a]Sekul, A.A. and W.C. Holland. 1961. Comparative pressor effects of certain unsaturated acid esters of choline. J. Pharm. Exptl. Therp. 133: 313-318.

preparative paper chromatography in system 25 (butanol:ethanol:water: acetic acid, 8:2:3:1, v/v, no KCl). Upon UV analysis, both components exhibited peaks at 203 and 225 nm although the faster moving component had at least a ten fold greater absorbtivity. Hydrolysis of both components and subsequent GLC analysis of their volatile hydrolysis products on Porapak Q showed that the slower moving component released acrylic acid upon hydrolysis while the faster moving component released a negligible amount of acrylic acid and no other hydrolysis product detectable on the Porapak Q column. It was therefore concluded that the slower moving component of the synthesis product was, indeed, acrylylcholine while the faster moving component of the synthesis product was either a polymerization product of acrylic acid or acrylylcholine.